Annexin A1 as an endogenous regulator of mast cell degranulation

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I, Ajantha Sinniah, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated and appropriately referenced**.**

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ABSTRACT

Annexin A1 (Anx-A1) is a 37kDa protein that is secreted by some cells in response to glucocorticoids (GCs) and which mediates several of their acute anti-inflammatory effects. In addition to GCs, 'mast cell stabilising' cromones such as nedocromil also mobilise Anx-A1 by promoting its phosphorylation by protein kinase C (PKC) and hence its secretion, which explains their acute efficacy as anti-allergic agents. This thesis addresses a fundamental aspect of Anx-A1 in the actions of anti-allergic drugs. In this study, anti-allergic drugs such as H_1 antagonists, mast cell stabilisers and 'dual action' drugs were first tested for their ability to enhance Anx-A1 phosphorylation in a model system using U937 cells.

Biochemical and immuno-fluorescent techniques were used to study the mechanisms by which these drugs suppress mediator release from cord blood derived mast cells (CDMCs) and murine bone-marrow derived mast cells (BMDMCs) from wild type and Anx-A1 null-mice. This thesis suggest that PKC activation is crucial for Anx-A1 export in mast cells and nedocromil in the presence of dexamethasone, prolongs the duration of PKC activation and subsequently phosphorylation, externalisation and release of Anx-A1 from CDMCs. The ability of nedocromil to inhibit β-hexosaminidase, tryptase, histamine and PGD_2 release are dependent on Anx-A1 in CDMCs. Interestingly, ketotifen, a 'dual action' drug possesses a similar pharmacological profile to nedocromil, but not promethazine, which does not

act through the Anx-A1 release. Strong evidence supports the notion that the mechanisms of action of nedocromil are modulated by Anx-A1, thus the possibility that FPR2 might be involved in the acute actions of nedocromil was tested. Nedocromil inhibits the release of PGD_2 through the activation of FPR2 but not the inhibition of histamine release. A possible explanation for this finding could be that Anx-A1 might be interacting with other FPR family members to exert the histamine inhibitory effects. Although only a small subset of the downstream intracellular signaling pathway of MAPK was tested, the results indicate that Anx-A1 differentially regulates the activation of p38 and JNK in CDMCs treated with nedocromil.

These findings indicate a novel model system in which Anx-A1 mediates the pharmacological actions of anti-allergic drugs and thus has an important role in preventing the mast cell degranulation.

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Abbreviations

5-HT	5-hydroxytryptamine
ABC	ATP-binding cassette
AChE	acetylcholinesterase
ACTH	adenocorticotrophic hormone
Ala^{27}	alanine ^{27}
ANOVA	analysis of variance
Anx	Anexin
APS	ammonium Persulphate
ATP	adenosine triphosphate
AVP	arginine vasopressin
BAL	bronchoalveolar lavage
BALB/C	Bagg albino
BMDMCs	bone marrow derived murine mast cells
Boc-MLF	butyloxycarbonyl-Met-Leu-Phe
BSA	bovine serum albumin
BTK	Bruton's tyrosine kinase
c-src	sarcoma (proto-oncogene tyrosine-protein kinase)
CD	cluster of differentiation
CDMCs	cord-blood derived mast cells
CO_2	carbon dioxide
COPD	Chronic Obstructive Pulmonary Disease

COX-1	cyclooxgenase
$cPLA_2$	cytosolic phospholipase A_2
CRH	corticotrophin-releasing hormone
CTMC	connective tissue mast cell
DAG	diacylglycerol
DAMPS	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
ddH_2O	double distilled water
dH_20	distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMF	N-N-dimetyhlformamide
DMSO	Dimethyl suphoxide
DNA	Deoxyribonucleic acid
DNP	Dinitrophenyl
DP	Prostaglandin D_2 receptor
ECL	enhanced chemiluminesscene
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth factor
EGF-R	Epidermal Growth factor receptor
ELISA	Enzyme linked immunosorbent assay
ERK	extracellular signal regulated kinase
FACS	Fluorescence-activated cell sorting

FBS	Fetal Bovine Serum
FGF	Fibroblast growth factor
FPR	formyl-peptide receptors
FPR1	formyl-peptide receptors 1
FPR2	formyl-peptide receptors 2
FPR3	formyl-peptide receptors 3
Gads	GRB2-related adapter protein
GAG	glycosaminoglycan
GC	Glucocorticoid
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G-protein coupled receptors
GR	Glucocorticoid receptor
GRB2	Growth factor receptor-bound protein 2
GRE	Glucocorticoid response elements
GTPyS	guanosine 5'-O-[gamma-thio]triphosphate
GWAS	genome-wide association studies
H	Histamine
H_20_2	hydrogen peroxide
HDAC	histone diacethylases
HDC	histidine carboxylase
hFPR1	human formyl-peptide receptors 1
hFPR3	human formyl-peptide receptors 3

HGF	hepatocyte growth factor
hGR	human glucocorticoid receptor
HMGB1	high- mobility group box 1
HPA	hypothalamus-piyuitary axis
HRP	horseradish peroxidase
hsp90	heat shock protein
HUVECS	Human Umbilical Vein Endothelial Cells
ICAM-1	intracellular adhesion molecule
Ig	Immunoglobulin
IL	interleukin
IP3	inositol-1,4,5-triphosphate
ITAM	immunoreceptor tyrosine-based activation motif
JNK	c-jun N-terminal kinase
KO	knock-out
LAT	linker of activated T cells
LT	leukotriene
LXA4	lipoxin A4
M1	macrophage 1
M2	macrophage 2
mAb	monoclonal antibody
mACh	muscarinic acetylcholine
MAP	mitogen-activated protein

MAPK	mitogen-activated protein kinase
MC-CPA	mast cell carboxypeptidase A
MMC	mucosal mast cell
mMCP-6	mast cell-restricted tryptase
mRNA	messenger Ribonucleic acid
Na_2CO_3/	
$NaHCO_3$	Sodium carbonate/Sodium bicarbonate
Na_3VO_4	Sodium orthovanadate
NaCl	Sodium Chloride
NF-Kb	nuclear factor-kappa activated B
NSB	non specific binding
NSF	N-ethylmaleimide-sensitive factor
PAF	platlet activating factor
PAMPs	Pathogen Associated Molecular Patterns
PBS	Phosphate buffered saline
PFA	paraformaldehyde
PG	prostaglandin
PGD_2	prostaglandin D_2
PGF_2	prostaglandin F_2
PH	pleckstrin homology
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate 2

PKC	Protein Kinase C
PLA_2	Phospholipase A_2
PLCy	Phospholipase gamma
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PMN	polymophonuclear
PMSF	phenylmethanesulfonylfluoride
PP2A	phosphatase 2A
PRR	Pattern-recognition receptors
PS	phophatidylserine
PVDF	Polyvinylidene fluoride
RACKS	Receptors for activated-C kinases
RBL-2H3	rat basophilic leukemia
RNA	Ribo Nucleic Acid
RNS	reactive nitrogen species
ROS	Reactive Oxygen Species
RU486	Mifepristone
SAA	serum amyloid A
SAC	seasonal allergic conjunctivitis
SCF	stem cell factor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error mean

Ser^{27}	serine ^{27}
Ser^{45}	serine ^{45}
SNARE	soluble NSF attachment protein receptor
TEMED	tetramethylethylenediamine
TGF	Transforming growth factor
Th2	T-helper 2
TLRs	Toll-like receptors
TNF	tumour necrosis factor
TPH	tryptophan hydoxylase
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
TRPM7	Transient receptor potential cation channel, subfamily M, member 7
TTBS	Tween 20-Tris Buffered Saline
TxB_2	thromboxane B_2
Tyr^{21}	tyrosine 21
U937	pro-monocytic cell line
VAMP-8	vesicle associated membrane protein
VEGF	Vascular endothelial growth factor
WRW4	Trp-Arg-Trp-Trp-Trp-Trp-CONH2 4 peptide
WT	wild-type

1. INTRODUCTION

1.1 OVERVIEW OF THE INFLAMMATORY PROCESS

Inflammation may be defined as a constellation of rapid and coordinated process of adaptive response that is provoked by tissue damage or infection by pathogenic and non-pathogenic microbes (Barton, 2008). Although the cardinal signs of inflammation had been mentioned in ancient medical texts, the physiological basis of the clinical signs of inflammation was only revealed in 1846 by Augustus Waller who also discovered the leukocyte emigration into tissue (Medzhitov, 2010). The cardinal signs of inflammation are redness (*rubor*), heat (*calor*), swelling (*tumor*), pain (*dolor*) and eventually loss of function (*functio laesa*). These signs represent the macroscopic culmination of a myriad of molecular and cellular processes during inflammation.

A typical inflammatory response consists of four components, which are the inflammatory inducers, the sensors that detect them, the inflammatory mediators induced by the sensors, and the target tissues that are affected by the inflammatory mediators (Figure 1.1). The ideal inflammatory response eliminates the infectious agents and is followed by resolution and repair phase and a return to homeostasis. However, the orchestrated coordination of inflammation is dependent on the mechanism required to resolve inflammation, while limiting the damaging aspects of inflammation to the host tissue (Segal *et al.*, 2000; Nathan, 2002).

In many organs such as lung, skin and nose, intact epidermal or epithelial surfaces form the main interface between pathogens in the external

environment and host tissue. These interfaces are lined by continuous layers of specialised epithelial cells that serve as a physical barrier preventing the entry of microbes (Bulek *et al.*, 2010). There are two types of immunity that protects the host from infections, which are the innate and adaptive immune system. The innate system is a first line of defense as it acts as both a physical and chemical barrier to infectious agents (Janeway *et al.*, 2002). The innate system comprising macrophages, neutrophils, mast cell, basophils, eosinophils and dendritic cells, among others, is genetically programmed to detect the invariant features of invading pathogens. Even though the main immune cells in the brain are the microglial cells, recent evidences have indicated that neurons do express TLRs and that they detect intrinsic danger signals and modulate neuronal morphology and function in response to pathogen infection (Liu *et al.*, 2014). By contrast, the adaptive system comprising T and B-lymphocytes, are highly specific in that the generation of responses is tailor-made to maximise the elimination of specific inducers of inflammation. The adaptive immune system develops immunological memory of invading pathogens, which is 'remembered' by signature antibodies or T cell receptors. These memory cells will be immediately recruited to eliminate the pathogens should any subsequent pathogenic invasion occurs (Medzhitov, 2007).

An acute inflammatory response, depends upon the converging actions of various inflammatory mediators such as chemokines, cytokines, vasoactive amines and eicosanoids (Medzhitov, 2008). This conspicuous

event is initiated at the site of injury by the resident cells, notably macrophages and mast cells that secrete the pro-inflammatory mediators, which are considered pivotal for the progression of inflammation. These inflammatory mediators will provoke an inflammatory exudate locally by facilitating the infiltration of plasma proteins and neutrophils through the postcapillary venules to the extracellular tissue at the site of infection or injury (Pober *et al.*, 2007). Upon reaching the afflicted site of injury or infection, neutrophils will become activated either by direct contact with pathogens or through the actions of cytokines produced by tissue-resident cells. The neutrophil then attempts to destroy the invading agents by releasing the toxic contents of their granules, which includes reactive oxygen species (ROS), proteinase 3, cathepsin G and elastase (Nathan, 2006). However, these potent effectors are not able to distinguish between host targets and pathogens, thus collateral damage to the host tissue is often inevitable (Nathan, 2002). Interestingly, this equilibrium is really precise such that any modifications, through the release of enhanced pro-inflammatory factors would lead to cellular and tissue damage (Perretti *et al.*, 2009).

By contrast, chronic inflammation is a prolonged and dysregulated response that involves active inflammation, tissue destruction and attempts at tissue repair. As such persistent inflammation is often associated with many chronic human conditions and diseases, including atherosclerosis, arthritis, allergy, cancer and autoimmune diseases (Medzhitov, 2008).

Conventionally, it was thought that pro-inflammatory mediator catabolism was sufficient for inflammation to ‘switch off’ and the response would subsequently just finish ‘spontaneously’. However, we are now aware that the next concomitant event is cell clearance. For the inflammation to resolve, the inflammatory leukocytes need to exit through the available routes including systemic circulation or are removed by apoptosis followed by phagocytosis by macrophages, which would then egress from the inflamed site by lymphatic drainage (Davies *et al.*, 2013). The acute inflammation phase would then resolve without causing excessive tissue damage provided all these pathways are exactly adhered.

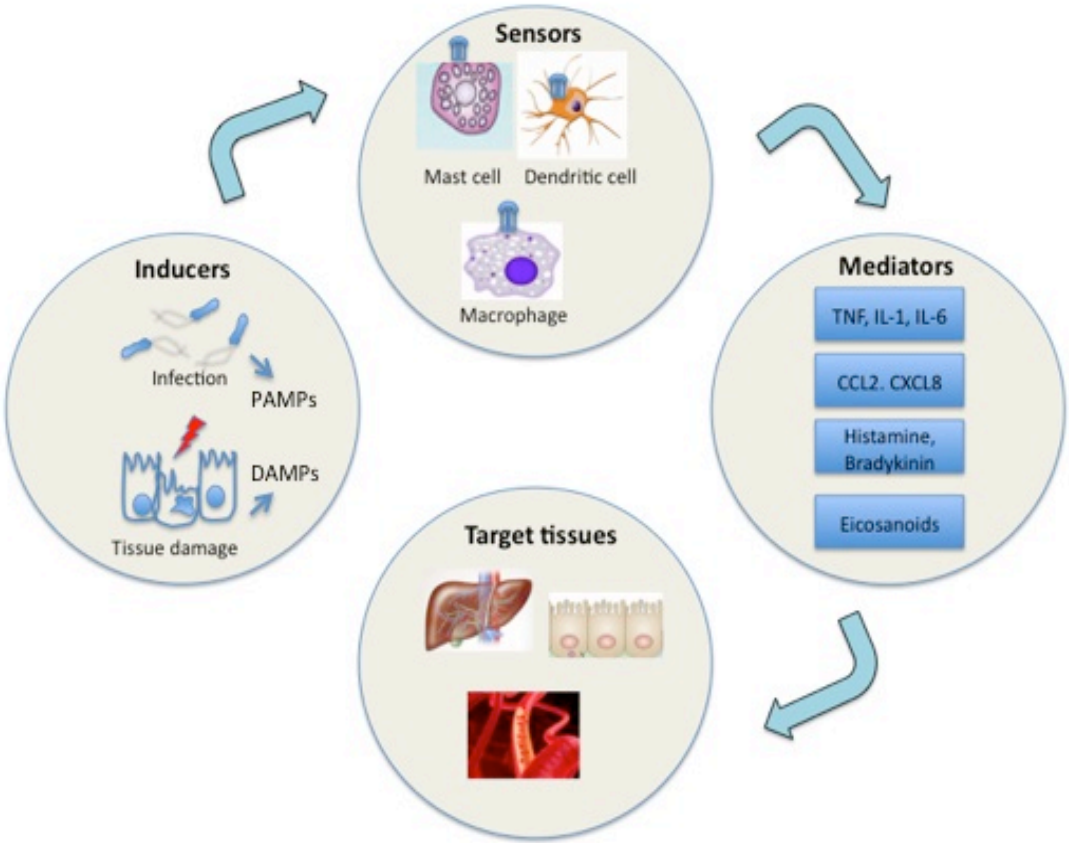


Figure 1.1: The inflammatory pathway consists of inducers, sensors, mediators and target tissues.

Upon exposure to pathogens or injury, pathogen associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are recognised by sensors such as Toll-like receptors (TLRs), which are expressed on macrophages, dendritic cells and mast cells. These cells induce the production of mediators, including cytokines, chemokines, eicosanoids, bioactive amines and products of proteolytic cascades, such as bradykinin. These inflammatory mediators act on target tissues to modify the functional state to maximise adaptation to injury or infection that initiated the inflammatory process (Medzhitov, 2010).

1.1.1 Inducers and sensors of inflammation

The innate immune system engages with invariant pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs) that recognise evolutionarily conserved structures such as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Akira *et al.*, 2006). In contrast, the adaptive immune system not only depends on the production of a repertoire of antigen receptors located on the T and B-lymphocytes but also strongly relies on the essential signals that are delivered by the innate the immune system (Schenten *et al.*, 2011). The crosstalk between innate and adaptive system determines the nature and duration of inflammation (Iwasaki *et al.*, 2010).

Inducers of inflammation could be classified as either exogenous or endogenous. Exogenous inducers of inflammation consist of microbial or non-microbial inducers. There are two further sub-classes of microbial inducers, which are PAMPs and virulence factors. PAMPs are a limited and conserved molecular patterns that is found in all microorganisms and there are receptors that specifically recognise PAMPs (Tang *et al.*, 2012). Therefore this evolutionary tactic of the host allows the adaptive immune response to discriminate between self and non-self. The second class of microbial inducer are virulence factors and one feature that distinguishes between PAMPs and virulence factors is that the latter are not sensed by receptors; therefore the adverse effects of their activity on host tissue are enough to trigger the inflammatory response (Medzhitov, 2008).

By contrast, damage-associated molecular patterns (DAMPs) are associated with cell damage and released in response to trauma, ischaemia or tissue damage, either in the absence or presence of pathogenic infection (Tang *et al.*, 2012). Many DAMPs are either nuclear or cytosolic proteins and when released from the cell, the oxidising extracellular milieu results in DAMPs denaturation (Rubartelli *et al.*, 2007). DAMPs include intracellular proteins, such as heat-shock proteins (Panayi *et al.*, 2004), high-mobility group box 1 (HMGB1) (Scaffidi *et al.*, 2002) and proteins derived from the extracellular matrix that are generated following tissue injury (Scheibner *et al.*, 2006). Whereas, non-protein DAMPs include ATP, uric acid, RNA and DNA. DAMPs also induce substances released from mitochondria, consisting of formyl peptides and mitochondrial DNA, which activate human polymophonuclear (PMN) neutrophils. It is noteworthy to mention that N-formylated peptides can be recognised by formyl-peptide receptors (FPR), which is also activated by the anti-inflammatory endogenous ligand, Anx-A1. Interestingly, PAMPs and DAMPs can share the same receptor, particularly the TLRs, indicating similarities do exist between pathogen-induced and non-infectious, inflammatory responses.

Non-microbial exogenous inducers include allergens, irritants, foreign bodies and toxic compounds. The inflammatory response provoked by allergens relies on expulsion and clearance mediated by the epithelial mucosa (Tillie-Leblond *et al.*, 2005). Foreign bodies such as silica and asbestos are indigestible particles that are either too big to be phagocytosed

by macrophages. These particles cause the generation of reactive oxygen species and reactive nitrogen species (ROS / RNS), which triggers signal transduction pathways that initiate inflammation (Fubini *et al.*, 2003; Shukla *et al.*, 2003).

1.1.2 Mediators and effectors of inflammation

The inducers of inflammation trigger the release and generation of various inflammatory mediators, which are the downstream effectors of the inflammatory pathway. Most of these mediators influence the vasculature and the recruitment of leukocytes. The cellular mediators are synthesised by specialised leukocytes such as macrophages or mast cells. Some mediators exist and circulate as inactive precursors in the plasma while others are preformed and stored in the granules of mast cells, basophils or platelets (Medzhitov, 2008).

Inflammatory mediators can be classified based on their biochemical properties. Vasoactive amines such as histamine and serotonin are produced when mast cells degranulate and they cause increased vascular permeability and vasodilation (Bach, 1982).

Other types of inflammatory mediators include vasoactive peptides, such as substance P, which are stored in an active form in the secretory vesicles or kinins, fibropeptide A and fibrin degradation products that are generated by inactive precursors in the extracellular fluid. Substance P is

released by sensory neurons and also cause mast cell degranulation (O'Connor *et al.*, 2004). Other vasoactive peptides are generated through proteolysis by thrombin or plasmin that cause vasodilation and increased vasopermeability. These vasoactive peptides affect the vasculature as well as triggering a pain sensation, which is one of the most important components of inflammation (Lühder *et al.*, 2009; Bhatia, 2010).

The third group of mediators of inflammation are the anaphylatoxins, which are produced by complement activation. These mediators promote granulocyte and monocyte recruitment, stimulate mast cell degranulation and affect the vasculature (Schmudde *et al.*, 2013).

Lipid mediators such as eicosanoids and platelet-activating factors are derived from phospholipids. Upon activation by intracellular Ca^{2+} ions, cytosolic PLA_2 generates arachidonic acid and lysophosphatidic acid. Arachidonic acid is metabolised to form eicosanoids either by cyclooxygenase, which generates prostaglandins and thromboxane (Scott *et al.*, 1999), or by lipooxgenases, which generate leukotrines and lipoxins (Serhan, 2006). The prostaglandins cause vasodilation, hyperalgesic and induce fever (Higgs *et al.*, 1984; Ivanov *et al.*, 2004). Meanwhile, lipoxin is an anti-inflammatory mediator, which inhibits inflammation and promotes the resolution of inflammation and tissue repair (Serhan, 2007). The second class of lipid mediator, which is platelet activating factor (PAF) is involved in platelet activation, recruitment of leukocyes, vasodilation and vasoconstriction and increased vascular permeability (Serhan, 2006). Stimulated basophils,

monocytes, PMNs, platelets, and endothelial cells produce PAF primarily through lipid remodeling. A variety of stimuli can initiate the synthesis of PAF, which could be either macrophages going through phagocytosis or the uptake of thrombin by endothelium cells. PAF could be synthesised by two different pathways, which includes *de novo* and the membrane-remodeling pathway. The former maintains the levels of PAF during normal cellular functions whilst inflammatory agents activate the latter pathway and it is thought to be the primary source of PAF under pathological conditions.

Inflammatory cytokines and chemokines are produced and secreted by many cell types. Cytokines activate the endothelium and induce an acute phase response (Cohen *et al.*, 1996) while the chemokines regulates leukocyte extravasation and chemotaxis towards the affected tissues (Zlotnik *et al.*, 2000; Moser *et al.*, 2004).

Lastly, proteolytic enzymes including elastin, cathepsin and matrix metalloproteinase have diverse roles in promoting inflammation such as tissue defense, tissue remodelling and leukocyte migration (Mithieux *et al.*, 2005; Le *et al.*, 2007; Conus *et al.*, 2010).

1.1.3 Homeostatic control through stress response and adaptation

Homeostasis is defined as a collection of control mechanisms that ensure that internal environmental parameters are maintained, within a normal equilibrium near a certain set point usually through a system of

feedback controls (Cannon, 1929). Abnormality in the homeostatic range could result in either acute stress response that causes a temporary adaptation to the new conditions or a more prolonged adaptive change that shifts the relevant set points (Medzhitov, 2008).

In the same vein, we can also appreciate that the acute and chronic inflammation are different types of adaptive response that occurs when the homeostatic mechanisms are insufficient. Acute inflammatory responses will resolve when the abnormal conditions are transient, thus functionality is restored and basal homeostatic checkpoints are maintained. However, when the abnormal conditions are sustained, the equilibrium shifts to extreme end of the spectrum resulting in chronic inflammation (Medzhitov, 2010).

1.1.4 Resolution of inflammation

Perpetuation of inflammation is an innate risk because it causes tissue damage that leads to scarring and loss of function. The paradox of inflammation is not how often it starts, but how often it fails to subside (Nathan *et al.*, 2010). Resolution of inflammation could be defined as an active endogenous program involving various biochemical processes and multiple signaling pathways that switch from the production of pro-inflammatory mediators to pro-resolving molecules such as lipid mediators and Annexin A1 (Anx-A1) that ensure rapid restoration of tissue homeostasis (Serhan *et al.*, 2007; Alessandri *et al.*, 2013).

At certain ‘checkpoints’ during the inflammatory process, appropriate ‘stop signals’, which include lipoxins and resolvins, are activated to prevent further leukocyte traffic into the tissue (Serhan *et al.*, 2008). These pro-resolving mediators pave the way for monocyte migration and differentiation into phagocytosing macrophages, which remove dead cells and then exit the site of inflammation (Ortega-Gómez *et al.*, 2013). There are two different subsets of macrophages, which are the classically activated M1 macrophages and the alternatively activated M2 macrophages. M1 macrophage mediates defence of the host from a variety of bacteria, protozoa and viruses, and has roles in anti-tumour immunity. In contrast, M2 macrophage have anti-inflammatory function and regulate wound healing. Fibroblasts contribute to the resolution of inflammation by withdrawing the survival signals and normalisation of the chemokine gradients, by allowing infiltrating leukocytes to undergo apoptosis or leave the tissue site through the draining lymphatics (Barone *et al.*, 2012). The events that governed the resolution phase are essential for complete resolution and restoration of inflamed tissue to ensure physiological functioning.

Currently, most therapies are targeted to inhibit the production of pro-inflammatory mediators, however an equally important target would be the initiation of endogenous pro-resolving mediators (Sousa *et al.*, 2013) . With the notion that inflammation is resolved in a time- and space- specific manner, elucidation of a certain pathway or mediator could lead to the

recognition of the molecular targets that could prompt novel drug discovery for the treatment of chronic inflammatory conditions (Serhan *et al.*, 2007).

1.2 ANX-A1 AS AN ENDOGENOUS ANTI-INFLAMMATORY PROTEIN

1.2.1 Annexin superfamily

The annexin superfamily in mammals consist of 12 calcium and phospholipid binding proteins with a 40-60% structural homology (Raynal *et al.*, 1994), and these are thought to derive from the ancestral Annexin A-13 (Anx-A13) gene (Gerke *et al.*, 2005). A common characteristic of annexins is their ability to bind to negatively charged phospholipid membranes in a calcium-dependent manner. The evolutionary difference between these subfamilies is the ability of these proteins to preferentially localise to accessible sites on the protein surfaces that determine membrane binding and the interaction with cytosolic proteins (Moss *et al.*, 2004).

A common trait of the family is a core domain, which normally comprises four conserved 70 amino acid motifs, which bear the calcium or phospholipid binding consensus sequence. The N-terminus which is thought to be responsible for the unique biological activity and specific functions of each member of the annexin superfamily, is of varying amino acid sequence and length (Gerke *et al.*, 2002).

Annexins are mainly localised in the cytosol either in a soluble form or

associated with S-100 proteins or components of the cytoskeleton (Moss *et al.*, 2004). Anx-A2 and Anx-A11 have been found in the nucleus under certain circumstances (Eberhard *et al.*, 2001; Tomas *et al.*, 2003). Anx-A1 was shown to be expressed on the cell surface despite the absence of any secretory signal peptide (Solito *et al.*, 1994) and Anx-A2 is constitutively expressed at the surface of vascular endothelial cells where it regulates blood-clotting mechanisms (Brownstein *et al.*, 2001). The tissue distribution and expression of these annexins range from being ubiquitous and abundant (Anx-A1, A2, A4, A5, A6 and A7) to selective (Anx-A3 in neutrophils and A8 in placenta and skin) or restrictive (A9 in tongue and A10 in the stomach).

The development of knock-out (KO) mice has provided immense understanding into the functions of annexins. Anx-A1 KO mice exhibit increased inflammatory responses and are resistant to glucocorticoid treatment (Hannon *et al.*, 2003) and the loss of Anx-A2 causes defects in neovascularisation and fibrin homeostasis (Ling *et al.*, 2004b). Anx-A7 null mutant mice strains show changes in calcium homeostasis (Herr *et al.*, 2001). This diversity of phenotype in the annexin KO mice suggests that these proteins have largely independent functions.

1.2.2 Structural characteristics of Anx-A1

Our laboratory pioneered the work on the 37kD endogenously expressed protein called Anx-A1, finding that this mediator is able to inhibit PGE_2 release by inhibiting the action of PLA_2 (Flower *et al.*, 1979). This protein was previously termed macrocortin (Blackwell *et al.*, 1980), renocortin (Russo-Marie *et al.*, 1982), lipomodulin (Hirata, 1981) or lipocortin 1 (Browning *et al.*, 1990). There were several other proteins with a homology to lipocortin and due to the ability of this protein to 'annex' phospholipid membranes, another name was agreed upon, Anx-A1 (Gerke *et al.*, 2002).

Anx-A1 was purified to homogeneity from peritoneal lavage fluid obtained from rats treated with GCs (Pepinsky *et al.*, 1986b), and subsequently cloned and sequenced in 1986 (Wallner *et al.*, 1986). Anx-A1 is found in many differentiated cells and tissues and it makes up 2-4% of the total cytosolic protein in the PMN although it is not normally detectable in the plasma membrane (Perretti *et al.*, 1996c). However, Anx-A1 is found in many tissues, including the lungs, bone marrow and intestine, at concentrations of < 50ng/ml, with the highest levels reported to be in the seminal fluid (150ug/ml). Anx-A1 comprises 346 amino acids and the N-terminal domain, which contains 41 residues (Rosengarth *et al.*, 2001a) contains several putative *Ser*, *Tyr* and *Thr* phosphorylation sites as well as consensus sequences for glycosylation, transglutamination and proteolysis (Raynal *et al.*, 1994). Crystallographic analysis of the Anx-A1 protein (Figure 1.2) suggests that the

alignment of the four repeated sequences creates a pore, giving the molecule a 'doughnut-like' appearance. It was disclosed that the N-terminus is embedded within the core domain on the concave surface, but increased Ca^{2+} concentrations (≥ 1mM in plasma or other biological fluids) causes the N-terminus region to 'flip out' and may thereby influence the biological activity of this protein (Rosengarth *et al.*, 2001a).

Anx-A1 is found mainly in the gelatinase granules of neutrophils (Perretti *et al.*, 2000), and α-granules of mast cells (Oliani *et al.*, 2000) whereas in the macrophages, it is found mainly within the cytoplasm although it is also associated with the membrane, cytoskeleton and nucleus depending upon the state of cell activation (Peers *et al.*, 1993; Seemann *et al.*, 1997). Anx-A1 is highly expressed by differentiated cells, as past studies have shown that Anx-A1 expression is lower in monocytes as compared to those in macrophages retrieved from the same donor (Perretti *et al.*, 1996c). Whilst, T-cells express Anx-A1, B cells only express it at low levels, whereas platelets do not express this protein at all (Cirino *et al.*, 1987; Morand *et al.*, 1995; Rescher *et al.*, 2004).

Work within our group has demonstrated that a peptide spanning the first 24 amino acids of Anx-A1, known as acetyl 2-26 (Ac2-26), mimics the anti-inflammatory effects of the human recombinant Anx-A1, indicating that this short region is responsible for the specific effects of the parent protein (Perretti, 1997).

Interestingly, Anx-A1 possesses proteolytic motifs and a N-terminal

truncated moiety which is commonly found in inflammatory fluids (Liu *et al.*, 2005) and the physiological and biological properties of Anx-A1 are destroyed by proteolysis of the N-terminal domain (Cirino *et al.*, 1993).

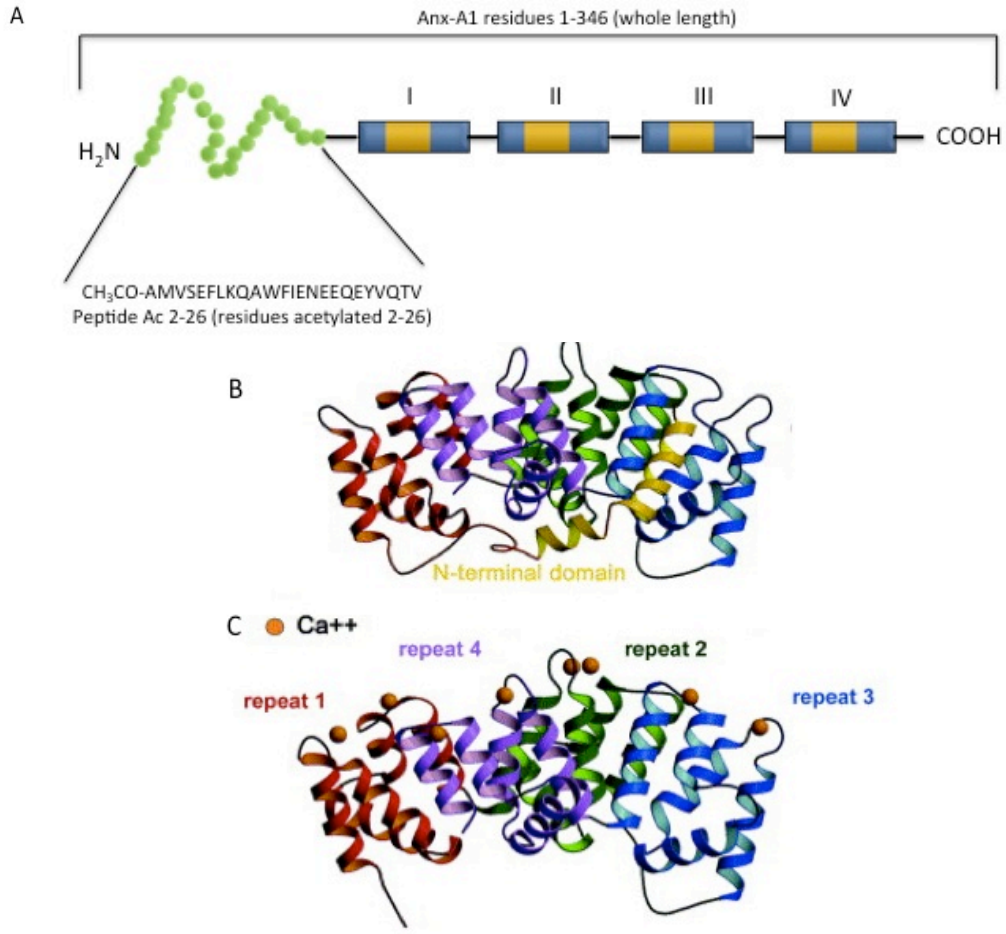


Figure 1.2: Anx-A1 peptide and molecular structure.

A) Schematic representation of Anx-A1 and peptides derived from the primary sequence. B) Ribbon presentation showing the 3-dimensional fold of Annexin A1 with four repeats and the N-terminal domain is folded into core domain on the concave surface. The N-terminal domain is shown in yellow, repeat 1 in red, repeat 2 in green, repeat 3 in blue and repeat 4 in purple. C) In the presence of Ca^{2+} ions (orange spheres), the N-terminal helix is exposed from the protein core and most likely becomes accessible for other interactions (Rosengarth *et al.*, 2003).

1.2.3 Post translational modification of Anx-A1

Many anti-inflammatory and other regulatory actions of Anx-A1 appear to be exerted extracellularly via membrane-bound cell surface receptors (Goulding *et al.*, 1992; Christian *et al.*, 1997; Gerke *et al.*, 2002; Perretti *et al.*, 2002). The availability of endogenous Anx-A1 to bind to these receptors is thus mainly dependent on the translocation of these proteins from its cytoplasmic storage sites to the cell surface. For example, within the neuroendocrine system, Anx-A1 is expressed in folliculostellate cells in regions adjacent to its target cells (Traverso *et al.*, 1999) and this protein is then translocated to the cell surface in response to appropriate stimuli (Loxley *et al.*, 1993b; Taylor *et al.*, 1993).

Substantial evidence supports the notion that secretion of Anx-A1 is an important event within the host defence system, for example in modulating neutrophil migration (Perretti *et al.*, 2002). However, since this protein does not possess a leader sequence or a secretory signal, the mechanism by which Anx-A1 is translocated from the cytoplasm to the cell membrane is not well understood. There maybe more than one pathway as secretion is independent from the classical endoplasmic reticulum/Golgi route of exocytosis (Philip *et al.*, 1998) and *de novo* protein synthesis (Solito *et al.*, 2003b). Moreover, in cells that do not store Anx-A1 in granules, secretion must be mediated through a separate mechanism. Some evidence does indicate that it is exported by the ATP-binding cassette (ABC) transporter system (Morris *et al.*, 2002; Chapman *et al.*, 2003).

Our past work has demonstrated that the externalisation of Anx-A1 from the cell requires a signalling cascade involving protein kinase C (PKC) leading to Anx-A1 phosphorylation prior to being rapidly mobilised to the outside leaflet of the plasma membrane (Yazid *et al.*, 2009). This post-translational modification occurs prior to the exportation of Anx-A1 out of the cell, however when Ser^{27} was mutated to Ala^{27}, there was no accumulation of Anx-A1 at the cell membrane further reiterating the role of Anx-A1 phosphorylation at Ser^{27} residue (Solito *et al.*, 2003c). At this location, the intact 37kDa Anx-A1 undergoes a conformational change, exposing the N-terminal region, resulting in a structure that is the active form of Anx-A1 (Rosengarth *et al.*, 2001b).

1.2.3.1 Phosphorylation of the N-terminal domain

The interactions between Anx-A1 and the cellular membrane are reversible and regulated by post-translational modification such as phosphorylation. The N-terminal domain of Anx-A1 contains several sites where post-translational modification occurs, and this process is fundamental for protein function (Table 1.1).

It has been previously reported that the N-terminal domain of Anx-A1 binds to the epidermal growth factor receptor (EGF-R) (Pepinsky *et al.*, 1986a) prior to phosphorylation at Tyr^{21} residue. This is crucial for the inhibitory effects of GCs on EGF-R, thus leading to a blockade of cytosolic

PLA_2 translocation to the membrane and inhibition of the release of arachidonic acid (Croxtall *et al.*, 2000). Anx-A1 was rapidly phosphorylated at tyrosine residue and translocated to the membrane fraction within 10 min of exposure to hepatocyte growth factor (HGF) in A549 lung carcinoma cells, suggesting that the phosphorylation of Anx-A1 may regulate cell proliferation, chemotaxis and vascular remodelling (Melki *et al.*, 1994). Anx-A1 expression and phosphorylation at Tyr^{21} residue was also implicated in liver regeneration and tumorigenesis either through modulating $cPLA_2$ activity or EGF-R function (de Coupade *et al.*, 2000). Interestingly, a study by Solito and co-workers using both sense and anti-sense Anx-A1 plasmid transfection in U937 cells, have demonstrated that the tyrosine phosphorylation is not essential for the blockade of cytosolic PLA_2 as only 10% of Anx-A1 is phosphorylated on the tyrosine residue (Solito *et al.*, 1998b). Intriguingly, Croxtall *et al* have demonstrated that the $cPLA_2$ and arachidonic release by the A549 human lung adenocarcinoma epithelial cell line was controlled by an *EQEYV* domain localised at the N-terminal region of Anx-A1, which comprises the Tyr^{21} phosphorylation motif (Croxtall *et al.*, 1998).

Dorovkov and colleagues have reported that the TRPM7 channel kinase (Chak1) is involved in Anx-A1 phosphorylation at the Ser^{5} residue (Dorovkov *et al.*, 2004). Another study had established an association between Anx-A1 and TRPM7 by confirming the presence of a TRPM7/Anx-A1/Mg^{2+} complex, suggesting a novel pathway for bradykinin signalling, which is dependent on PKC and c-src (Callera *et al.*, 2009). Anx-A1 phosphorylation

at *Ser*^{5} residue had also been shown to regulate the interaction between Anx-A1 and the calcium binding protein, S100A11 (Dorovkov *et al.*, 2011). This complex is considered to be biologically relevant as the disruption of this complex increased cervical cancer HeLa cells migration and clonogenic growth through the EGF signalling (Poeter *et al.*, 2013).

A key paper by John and colleagues has shown that PKC phosphorylates Anx-A1 and this event is crucial for the cellular export and biological activity of Anx-A1 in anterior pituitary glands and the Anx-A1 exported from these cells contains phospho-serine (John *et al.*, 2002). Following this lead, Solito *et al* demonstrated that dexamethasone induces rapid serine phosphorylation followed by membrane translocation of Anx-A1 via novel GC receptor-dependent mechanism, which is dependent on MAPK, PI3K and Ca^{2+}-dependent PKC pathway (Solito *et al.*, 2003c).

Anx-A1 also regulates hormone exocytosis, whereby the release of adrenocorticotrophin from a corticotroph-like cell line is inhibited by GCs. Furthermore, phosphorylation at *Ser*^{27} and *Ser*^{45} residues of Anx-A1 is crucial in the translocation of the protein to the cell membrane and inhibition of adrenocorticotrophin release involving the enhancement of actin (McArthur *et al.*, 2009). Work within our own group has shown that GC treatment of U937 cells induced a rapid concentration-dependent activation of PKCα/β prior to the Anx-A1-*Ser*^{27} phosphorylation, which precedes export of the protein. Cromoglycate-like drugs have minimal effects on the phosphorylation of either proteins, but greatly potentiate the effect of the GCs resulting in the

inhibition of thromboxane B_2 generation (Yazid *et al.*, 2009). Petrella *et al* have shown that the Ser^{27} phosphorylated form of Anx-A1 was exposed to the cell surface when an histone deacetylases (HDAC) inhibitor was used to induce apoptosis in U937, K562 and Jurkat leukemia cells, suggesting that Anx-A1 is present on the apoptotic cell surface and may be functioning as a putative pro-apoptotic signal (Petrella *et al.*, 2008).

These studies have roused attention on the regulatory role of post-translational modifications of Anx-A1, whereby the understanding of the mechanism of the structural switch on the phosphorylation of this protein could be fundamental in delineating the physiological functions of Anx-A1 especially the specific intracellular pathway and receptor interactions.

Kinase	Residue	Cellular effects	References
EGF-R	*Tyrosine*[21]	• Cell proliferation, chemotaxis, vascular remodeling • Liver regeneration and tumorigenesis • Inhibition of $cPLA_2$ and arachnidonic release	• Melki *et al*., 1994 • de Coupade *et al*., 2000 • Croxtall *et al*., 2000
Chak1	*Serine*[5]	• Binding with S100A11 • TRPM7/Anx-A1/Mg^{2+} bradykinin pathway	• Dorovkov *et al*., 2011 • Dorovkov *et al*., 2004
PKC	*Serine*[27]	• Translocation of Anx-A1 to membrane • Hormone exocytosis • Inhibition of TxB_2 • Induce pro-apoptotic signal	• John *et al*., 2002; Solito *et al*., 2006 • McArthur *et al*., 2009 • Yazid *et al*., 2009 • Petrella *et al*., 2008

Table 1.1: Principal cellular effects on the N-terminal domain of Anx-A1 phosphorylation of its respective residues.

1.2.3.2 Proteolytic regulation of N-terminal domain

Whilst there is much evidence supporting the need for N-terminal serine phosphorylation preceding Anx-A1 release, it is still unclear whether Anx-A1 externalisation requires N-terminus cleavage. Anx-A1 harbours several proteolytic motifs and an N-terminal truncated moiety, is commonly found in the inflammatory fluids. Thus, proteolysis of the N-terminal domain modifies the physical and physiological functions of Anx-A1.

Several enzymes cleave Anx-A1 into a 33kDa fragment, including elastase (Rescher *et al.*, 2006), metalloprotease (Movitz *et al.*, 1999) and proteinase 3 (Vong *et al.*, 2007). The purpose of this cleavage process is yet to be unravelled, although there have been studies reporting that the cleavage of the N-terminal domain occurs either to allow Anx-A1 to act as a pro-drug through the production of a bioactive fragment (Gavins *et al.*, 2003) or to limit the actions of Anx-A1 (Pederzoli-Ribeil *et al.*, 2010).

Moreover, a modified recombinant form of Anx-A1 known in our laboratory as the 'super' Anx-A1, which is resistant to the cleavage by the proteinase 3 enzyme have been shown to have longer lasting effects on neutrophil adhesion *in vivo* as compared to the native Anx-A1 (Pederzoli-Ribeil *et al.*, 2010).

1.2.4 Activation of N-formyl peptide receptors by Anx-A1

The way in which Anx-A1 mediates its cellular effects remained largely unknown until the year 2000. Goulding and co-workers postulated that exogenous Anx-A1 mediated its effects through specific cell surface receptors in peripheral blood monocytes and PMNs (Goulding *et al.*, 1996). However a breakthrough came through the work of Gerke and colleagues, demonstrating that formyl peptide receptor (FPR) antagonists blocked the anti-inflammatory effects of both the intact Anx-A1 and the Anx-A1-derived peptide Ac2-26, suggesting an important role for FPRs in regulating the functional effects of Anx-A1 (Walther *et al.*, 2000).

The anti-migratory effects of exogenous Anx-A1 were blocked by FPR antagonists, such as 'Boc' (butyloxycarbonyl) derivatives (Boc-MLF), on human neutrophils across a monolayer of endothelial cells. This effect was characterised by a transient calcium flux and L-selectin shedding (Walther *et al.*, 2000). These *in-vitro* data were supported by *in-vivo* studies from our own lab using the fpr2/3$^{-/-}$ mice, in which the inhibitory actions of Anx-A1 and its mimetics in a model of peritonitis were attenuated (Perretti *et al.*, 2001). These fundamental studies have opened up new avenues of research in the field of Anx-A1, paving way for further investigations into the possible functional and molecular links between Anx-A1 and this family of receptors.

FPR is a member of a seven-membrane spanning G-protein coupled receptors (Perretti *et al.*, 2002), which are expressed by several types of cells

including human neutrophils, monocytes, macrophages, mast cells, epithelial cells and endothelial cells (Serhan *et al.*, 2008). In human, there are three FPRs, which are termed FPR1, FPR2, and FPR3 (Ye *et al.*, 2009). FPR2 is otherwise known as FPR2/ALX as it is also the receptor for lipoxin A_4. On the contrary, the mouse Fpr gene family is more complex and contains at least eight related genes, including Fpr1, Fpr2, Fpr-rs1, Fpr-rs3, Fpr-rs4, Fpr-rs5, Fpr-rs6, Fpr-rs7, and Fpr-rs8 (Gao *et al.*, 1998; Ye *et al.*, 2009).

By sequence homology human and mouse families share two gene clusters. hFPR1 (human formyl peptide receptor 1) and mfpr1 (murine formyl peptide receptor 1) share ~77% homology whilst hFPR2 and mfpr2 genes, share approximately 76% homology, also share similarities with hFPR3 (Ye *et al.*, 2009).

The generation of fpr2/3 null mice, in which the murine homologue of FPR2 were deleted, has led to the greater understanding of the roles played by this receptor. This study reported that these mice were unable to respond to the N-terminal peptide Ac 2-26 and other agonists of the FPR2 receptor and exhibited an exacerbated inflammatory response when compared to the WT mice (Dufton *et al.*, 2010a). Intriguingly, these observations were found to be similar to Anx-A1 null mice (Hannon *et al.*, 2003).

FPR2 is a promiscuous receptor in that it recognises a repertoire of protein/peptides agonist as well as high affinity endogenous lipid ligands such as lipoxin A_4 (LXA_4) and resolvin D1 (Chiang *et al.*, 2000; Perretti *et al.*, 2002). This receptor not only transduces the anti-inflammatory effects of LXA_4

in many systems (Chiang *et al.*, 2006) but also mediates the pro-inflammatory responses to serum amyloid A (SAA) and other peptides (He *et al.*, 2003). The possibility that this receptor mediates two opposing effects could be due to the different binding site domains used by different agonists (Le *et al.*, 2005).

It was established in previous studies that LXA_4 and its analogs have contrasting effects to Anx-A1 and its mimetics even though both interact with the same receptor on peripheral leukocytes. LXA_4 causes increased basal surface expression of L-selectin (Gavins *et al.*, 2005b) whereas Anx-A1 and its mimetics cause L-selectin shedding by both neutrophils and monocytes in vitro (József *et al.*, 2002; Solito *et al.*, 2003a). These findings could be possible due to the ligand-specific conformational changes (Gavins *et al.*, 2005a) or the dimerisation state of the receptor (Cooray *et al.*, 2013) that yields specific cellular effects unique to each ligand.

An elegant study by Cooray and co-workers has shown that FPR2 homodimerisation displayed a higher tendency to signal in response to pro-resolving and anti-inflammatory agonist stimulation when compared to FPR1 and FPR3, whilst the pro-inflammatory agonist, serum amyloid A (SAA) did not share this feature. It is probable that the agonist-biased FPR2 dimerisation is able to discriminate between agonists with varying biological properties (Cooray *et al.*, 2013).

1.2.5 Anx-A1 as a mediator of GC action

Glucocorticoids (GC) induce the synthesis and secretion of Anx-A1 utilising both genomic and non-genomic pathways (Parente *et al.*, 2004). The underlying mechanism of GCs that regulate Anx-A1 gene transcription has not fully elucidated, it has been shown that even though the Anx-A1 promoter does not have a canonical sequence for GC response, it does have a binding site for IL-6, which suggest that GCs indirectly regulates Anx-A1 expression (Solito *et al.*, 1998a).

A substantial body of evidence shows that Anx-A1 is indeed an endogenous protein mediating many anti-inflammatory effects of GC. COX-2 mRNA and protein levels were constitutively increased in Anx-A1 null mice and more importantly, GCs were ineffective in models of inflammation (Hannon *et al.*, 2003), reiterating the homeostatic anti-inflammatory ability of Anx-A1 to resolve inflammation.

An intricate interplay between Anx-A1 and corticosteroid hormones was discovered when it was observed that Anx-A1 modulates GC-induced secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland (Loxley *et al.*, 1993a; Taylor *et al.*, 1993). Anx-A1 levels in murine peripheral blood leukocytes were found to be elevated 2-3 fold with a peak at 2 h after steroid treatment and the GC receptor antagonist, RU486, blocked this effect (Perretti *et al.*, 1996c).

During the inflammatory process, GCs cause rapid non-genomic mobilisation and secretion of Anx-A1 at the cell surface, and a slower (2-4 h)

upregulation of Anx-A1 gene transcription through genomic mechanisms (Croxtall *et al.*, 2000). The way by which GCs inhibit eicosanoids could be grouped into two main mechanisms based on Anx-A1 dependency. Firstly, rapid exposure of GCs prevent the phosphorylation or activation of $cPLA_2$ through Anx-A1 dependent mechanism (Croxtall *et al.*, 1996), and secondly, a more delayed exposure of these drugs down-regulates Cox-2 mRNA through an Anx-A1 independent mechanism (Masferrer *et al.*, 1992).

Exogenous recombinant Anx-A1 mimics corticosteroid suppression of monocyte functions such as superoxide generation (Maridonneau-Parini *et al.*, 1989) and autoimmune T lymphocyte proliferation (Gold *et al.*, 1996). An impaired induction of Anx-A1 by GC in monocytes (Morand *et al.*, 1995) and lowered Anx-A1 binding capacity in both neutrophils and monocytes were observed in rheumatoid arthritis (Goulding *et al.*, 1992). Failure of this regulatory system may be due to a defective HPA axis response to inflammatory or autoimmune injury resulting in reduced Anx-A1 expression. Anx-A1 is also involved in the anti-pyretic actions of GCs in rabbits (Davidson *et al.*, 1991) and in the GC inhibiting effect of hyperalgesia mediated by COX-2 induction in rats (Ferreira *et al.*, 1997).

Interestingly, clinical data also shows that leukocytes from patients with Cushing's disease, which is associated with higher cortisol levels, have elevated intracellular Anx-A1, whilst patients with Addison's disease, which is associated with lower cortisol levels have decreased intracellular levels of Anx-A1 in comparasion with healthy controls, suggesting that levels of Anx-

A1 is mediated by GCs during disease (Mulla *et al.*, 2005). Collectively, GCs have been shown to positively regulate the concentrations of Anx-A1, thereby assuring an appropriate level of activation of innate immune cells while limiting the duration of the pro-inflammatory response.

1.2.6 Anti-inflammatory effects of Anx-A1

Anx-A1 exerts anti-inflammatory effects in many experimental settings (Table 1.2). Anx-A1 was first discovered in an *in vitro* experiment whereby dexamethasone was shown to rapidly reduce the release of arachidonic acid through the inhibition of PLA_2 and COX-2 enzyme activity, which was dependent on the synthesis of a mediatory protein (Blackwell *et al.*, 1980; Hirata *et al.*, 1980). These actions of Anx-A1 seem to mimic the activity of aspirin. However, it is important to note that in asthmatic patients, aspirin provokes pro-inflammatory symptoms such as bronchoconstriction in the lung (Lee *et al.*, 2011). Since some reports have demonstrated that Anx-A1 is anti-inflammatory in allergic inflammatory models (Teixeira *et al.*, 1998; Bandeira-Melo *et al.*, 2005; Ng *et al.*, 2011; Wang *et al.*, 2011), this leads to the question on how Anx-A1 is anti-inflammatory in the lungs. A recent literature shows that Anx-A1 down-regulates ERK and NF-κB activity, which leads to the inhibition of TNF-induced proliferation and inflammatory responses in lung fibroblast (Jia *et al.*, 2013).

Anx-A1 was also reported to inhibit the synthesis of eicosanoid

mediators such as prostaglandins, leukotrines and PAF in *in vivo* model (Fradin *et al.*, 1988; Peers *et al.*, 1993). The role of the endogenous GC mediated protein, Anx-A1 was further confirmed via the use of DNA antisense oligonucleotides (Croxtall *et al.*, 1994; Perretti *et al.*, 1996a; Solito *et al.*, 1998b) or by the administration of human recombinant Anx-A1 (Errasfa *et al.*, 1989) and the KO mice.

Anx-A1 plays an important role in regulating neutrophil adhesion and transmigration (Perretti *et al.*, 1996b), which are the basis for the development of inflammation. The precise role of Anx-A1 during leukocyte transmigration has been defined using intravital microscopy techniques. Anx-A1 promotes L-selectin shedding in the neutrophils and induces detachment of the adherent leukocytes in the endothelium (Strausbaugh *et al.*, 2001). These actions are crucial to restrict leukocyte transmigration and recruitment during inflammation. An important observation reported by Hayhoe and colleagues was that full length Anx-A1 had no effect on PMN capture or rolling on TNF-α stimulated HUVECS but instead had an effect on cell adhesion (Hayhoe *et al.*, 2006). Anx-A1 also possess anti-migratory effects, whereby it reduces the α4β1 integrin-dependent monocyte adhesion and migration (Côté *et al.*, 2010). However Anx-A1 may produce more than one effect as Williams *et al*., have shown that activated neutrophils release a cleaved product from the C-terminus of Anx-A1, which acts to promote neutrophil transmigration by inducing the release of intracellular adhesion molecule (ICAM-1) on the endothelial surface (Williams *et al.*, 2010). In a

zymosan-peritonitis model, the Anx-A1 null mice displayed a higher degree of PMN recruitment, which was found to enhance cell emigration using intravital microscopy as compared to the wild type mice (Chatterjee *et al.*, 2005). Neutrophils isolated from Anx-A1 null mice and the WT cells treated with neutralising anti-Anx-A1 monoclonal antibody (mAb), exhibited hyper-reactivity to stimuli such as chemotactic factors (Chatterjee *et al.*, 2005), leading to increased superoxide radical release (Perretti *et al.*, 1995). Collectively these data indicate that the anti-inflammatory effects of Anx-A1 on PMNs may be attributed to their ability to enhance leukocyte detachment and inhibit emigration.

Several studies have indicated that Anx-A1 is involved in neutrophil apoptosis, thus limiting the inflammatory responses (Solito *et al.*, 2003a; Ishido, 2005; Petrella *et al.*, 2008). Specific 'eat me' signals on apoptotic cells contribute as markers for phagocytes to recognise and ingest as a means of clearing cell debri. The hallmark of apoptosis is the exposure of phophatidylserine (PS) on the outer leaflet membrane and this action inhibits the release of pro-inflammatory cytokine, TNF-α, although Il-1β stimulates the release of anti-inflammatory cytokines such as TGF-β and IL-10. Past studies have highlighted the possibility that Anx-A1 might serve as an endogenous PS ligand, thus mediating the engulfment of apoptotic cells, by demonstrating that Anx-A1 is recruited to the PS-rich region of apoptotic surface in a caspase-dependent mechanism and involves the release of intracellular calcium (Arur *et al.*, 2003).

The anti-inflammatory effects of Anx-A1 (Figure 1.3) counteract the inflammation and ensure a prompt resolution. The complete understanding of the effects of Anx-A1 should aid the development therapeutic drugs for the resolution of inflammation of both acute and chronic diseases.

System	Anti-inflammatory effects	References
In vitro	• ↓ $cPLA_2$ activation • ↓ Eicosanoid production • ↓ Superoxide generation • ↓ Phagocytosis • ↓ Histamine release • ↑ L-selectin shedding • ↑ PMN apoptosis	• (Solito *et al.*, 1998b) • (Yazid *et al.*, 2009) • (Perretti *et al.*, 1995) • (Becker *et al.*, 1988) • (Yazid *et al.*, 2013) • (Parente *et al.*, 2004) • (Solito *et al.*, 2003a)
In vivo	• ↓ PMN trafficking • ↓ histamine release • ↓PMN adherence • ↓cytokine generation • ↓ hyperalgesia/nociception • ↓fever • ↑ cerebral protection	• (Getting *et al.*, 1997; Chatterjee *et al.*, 2005) • (Bandeira-Melo *et al.*, 2005) • (Perretti *et al.*, 1996b) • (Loxley *et al.*, 1993b) • (Ferreira *et al.*, 1997) • (Davidson *et al.*, 1991) • (Gavins *et al.*, 2007)

Table 1.2: Some anti-inflammatory actions of Anx-A1 in both *in-vitro* and *in-vivo* settings.

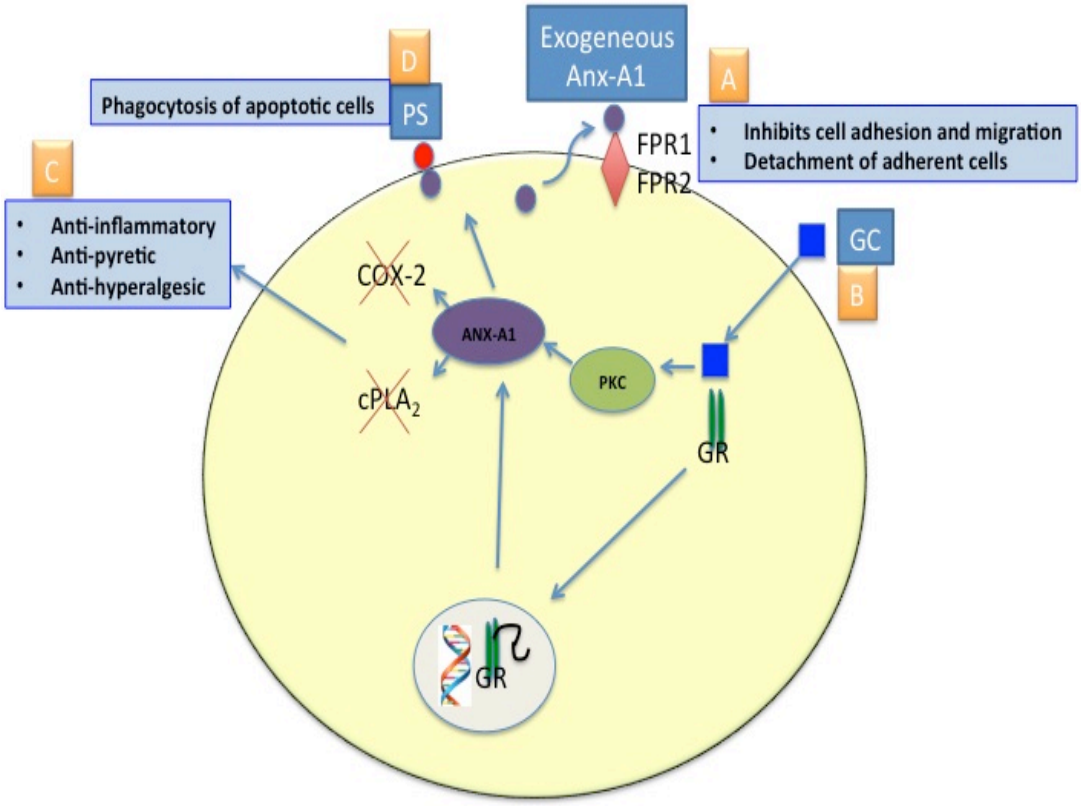


Figure 1.3: The principal mechanisms of Anx-A1 anti-inflammatory actions.

A) Exogenous Anx-A1 binds to formyl peptide receptors (FPR) to inhibit cell adhesion, migration and induce detachment of adherent cells. B) Anx-A1 expression and release is up-regulated with GC treatment through the GC receptor (GR) either through genomic or non-genomic mechanisms, which contributes to the anti-inflammatory effects of Anx-A1. GCs induce rapid Anx-A1 phosphorylation via the activation of PKC and initiate the membrane translocation of Anx-A1 molecule. C) Anx-A1 inhibited the cytosolic phospholipase A_2 ($cPLA_2$) and cyclooxygenase 2 (COX-2), thus exhibiting anti-inflammatory, anti-pyretic and anti-hyperalgesic activity. D) Anx-A1 is recruited to the cell surface, where it binds to phosphatidylserine (PS) and mediates the phagocytosis of apoptotic bodies (Lim *et al.*, 2007).

1.2.6.1 Tools developed to study the role of Anx-A1

Experiments using neutralising anti-Anx-A1 monoclonal antibody have provided substantial evidence that Anx-A1 is a key mediator of GC action in both the innate and adaptive immune systems. The acute inflammatory response in mice was exacerbated and did not respond to GC treatment in the presence of neutralising anti-Anx-A1 monoclonal antibody (Perretti *et al.*, 1996a). Other reports have demonstrated that the neutralising anti-Anx-A1 monoclonal antibody reversed GC-mediated inhibition of leukocyte migration in mouse peritonitis and air pouch models (Teixeira *et al.*, 1998) as well as the anti-inflammatory effects of GC in a rat carrageenin oedema model (Duncan *et al.*, 1993) and the GC-induced growth arrest in A549 lung adenocarcinoma cell line (Croxtall *et al.*, 1992). Anti-sense RNA to Anx-A1 has also been employed to explore the Anx-A1 mediated anti-inflammatory effects of GC providing data consistent with the above interpretation (Croxtall *et al.*, 1994; Taylor *et al.*, 1997).

Interest in exploiting Anx-A1 as a potential therapeutic target stemmed from the fact that administration of exogenous Anx-A1 rescues the inflammatory conditions in vivo models of ischaemia reperfusion (Gavins *et al.*, 2003). Human recombinant Anx-A1 has been shown to exert anti-inflammatory effects in a carrageenan-induced edema model of inflammation (Wu *et al.*, 1995). Following the discovery that the biological activity of Anx-A1 could be replicated by the first 26 amino acids of the N-terminus (N-acetyl 2-26), this peptide is commonly used instead of the full-length recombinant

molecule (Cirino *et al.*, 1993). The anti-inflammatory effects of both the human recombinant Anx-A1 and peptide Ac2-26 have been demonstrated in many models such as ischaemia/reperfusion injury (D'Amico *et al.*, 2000; La *et al.*, 2001), mouse air-pouch and rat edema models of inflammation (Cirino *et al.*, 1993; Perretti *et al.*, 1993), allergic inflammation models (Bandeira-Melo *et al.*, 2005), neutrophil and monocyte trafficking models (Szabó *et al.*, 1997) and pleurisy model in rats (Teixeira *et al.*, 1998).

The generation of Anx-A1 null mice by our laboratory has enabled researchers to study the functional and crucial role of this protein. The Anx-$A1^{-/-}$ mouse line was generated by homologous recombination, with a transgenic gene that disrupted the Anx-A1 gene, and inserted LacZ gene under the control of the Anx-A1 promoter (Hannon *et al.*, 2003). Mice lacking the Anx-A1 gene have been reported to have overzealous inflammation with an increase in COX-2 and PLA_2 expression (Roviezzo *et al.*, 2002) and are not responsive to GCs (Croxtall *et al.*, 2003). Anx-$A1^{-/-}$ mice have increased inflammatory responses as they display higher levels of inflammatory markers in joints (Reddy *et al.*, 2010), increased leukocyte transmigration (Chatterjee *et al.*, 2005), increased neutrophil recruitment in peritonitis model of inflammation (Damazo *et al.*, 2006), delayed resolution in a model of colitis (Babbin *et al.*, 2008) and increased neurological deficit in stroke (Gavins *et al.*, 2007). In particular relevance to this thesis, mast cells from the Anx-$A1^{-/-}$ mice were shown to exhibit histological signs of constitutive activation and increased basal mediator release (Lloret *et al.*, 1994).

To further elucidate the role of FPR in the mechanisms of Anx-A1, fpr2/3 null mice were generated (Dufton *et al.*, 2010a). Inflammation was more marked in the fpr2/3$^{-/-}$ mice with a more pronounced increase in cell adherence and emigration in the mesenteric microcirculation following ischaemia/reperfusion injury. In the model of carrageenan-induced paw edema, an increased acute response was observed in the fpr2/3 null mice in relative to the findings from the WT controls. Fpr2/3$^{-/-}$ mice also displayed a higher sensitivity to arthrogenic serum and this chronic pathology did not resolve over time (Dufton *et al.*, 2010a), suggesting that indeed fpr2/3 is a key receptor that mediates a variety of functions during host defense response.

1.3 GLUCOCORTICOIDS (GC)

One anti-inflammatory endogenous mediator, hydrocortisone (cortisol) and its synthetic analogues that have been widely used therapeutically in chronic inflammatory diseases (Barnes, 2011a). Despite the great benefits, GCs also incur a heavy burden of side effects over a prolonged exposure or in inappropriate doses. Nevertheless, GCs remain widely employed in the management of many inflammatory and autoimmune diseases. GCs activate many anti-inflammatory genes and repress many pro-inflammatory genes that are activated in inflammation (De Bosscher, 2010).

The main endogenous GCs are cortisol and corticosterone in mammalian species, however the amount produced varies amongst species. Cortisol is the predominant GC found in man whereas rodents produce corticosterone. Increased levels of cortisol dampen local and systemic inflammatory events during inflammation, thereby favouring proper resolution of the inflammatory response (Rhen *et al.*, 2005).

GCs have been described as 'anti-defensive' hormones that are released during any situation that threatens to compromise the homeostatic functioning of the body (Munck *et al.*, 1984; Sapolsky *et al.*, 2000). Correctly timed release of GCs is essential for human to respond and recover from stress, inflammation, injury or infection. This stress response is independent of the existing circadian cycle and varies in magnitude according to the nature, intensity and duration of the stimulus (Buckingham *et al.*, 1996).

The hypothalamus-pituitary axis (HPA) governs the circadian and

stress-induced secretion of GCs. The hypothalamus receives and integrates neural and humoral information, and thus acts as a sensor to monitor changes in the external and internal environment (Figure 1.4). The hypothalamus responds to these changes by activating the final common pathway to stimulate the synthesis of GC. Two hypothalamic neurohormones, corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) are released into the the portal blood supply and act synergistically via specific receptors to trigger the release of adrenocrticotropic hormone (ACTH) from the corticotrophs of the anterior pituitary gland into the systemic circulation. ACTH then acts on the adrenal cortex to initiate the synthesis of cortisol or corticosterone, which is released into the systemic circulation. Since the sensitivity of the HPA to incoming stimuli is regulated by a negative feedback mechanism, the magnitude of HPA response to stress depends upon the pre-existing GC in the circulation (Buckingham *et al.*, 1977).

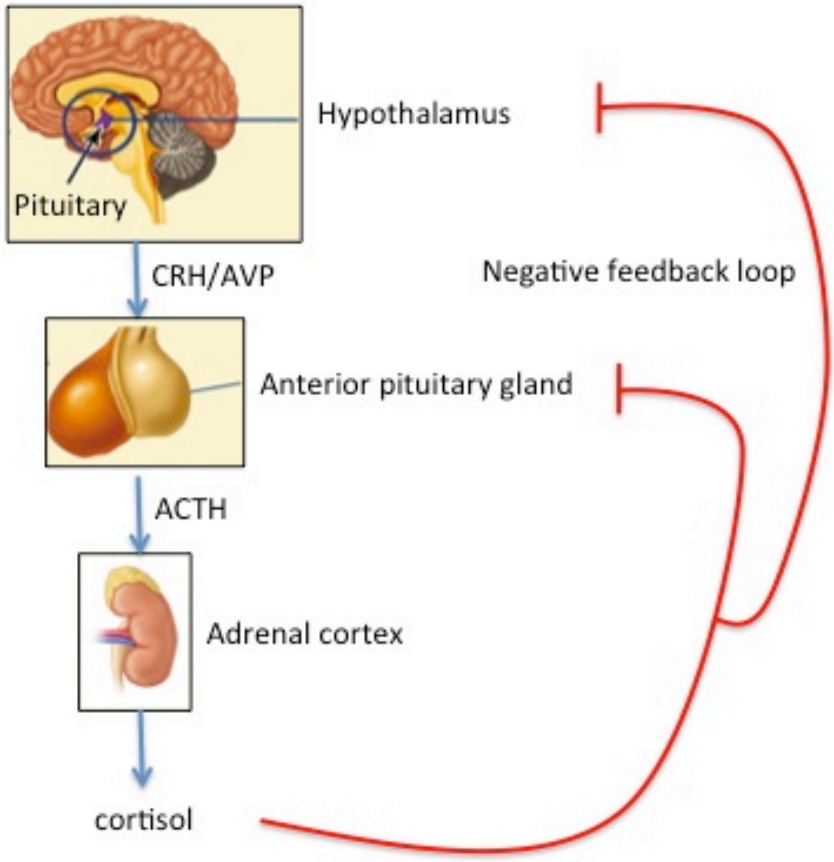


Figure 1.4: Interaction networks between HPA axis and the immune system via hormonal signals.

The HPA axis regulates the function of the immune system by releasing ACTH and cortisol/cortisone. GCs play an important end point role in the neuroendocrine system in regulating the network and affecting immune cells and molecules (Adapted from (Kadmiel *et al.*, 2013).

1.3.1 Glucocorticoid receptors (GR)

GCs exert pleiotropic effects on many target cells, tissues and organs through specific cytoplasmic and nuclear receptors known as GR. There are two different isoforms that have been cloned, hGRα and hGRβ (Encío *et al.*, 1991). While closely related to hGRα, hGRβ isoform is located in the nucleus and acts as a natural dominant negative inhibitor of hGRα (Kadmiel *et al.*, 2013). Although hGRβ lacks the GC binding domain (Oakley *et al.*, 1996), it has been reported that GR antagonist, RU486 (mifepristone) binds with hGRβ and regulates its transcriptional activity (Lewis-Tuffin *et al.*, 2007)

hGRα resides predominantly in the cytosol but upon GC binding, it translocates into the nucleus to play a role as a transcription factor (Adcock *et al.*, 2004) or by initiating intracytosolic signal transduction cascades (Solito *et al.*, 2003c). In the absence of GC, hGRα exists as a large heteromeric complex consisting of the receptor polypeptide, two molecules of hsp90, and one molecule of the hsp56 (Nicolaides *et al.*, 2010). The association of hGRα with hsp90 maintains the receptor in a conformation that will not bind to DNA, but will bind to the GC with high affinity. Whereas, the hsp56 which associates with GRα-hsp90 complex, has an important chaperone role (Renoir *et al.*, 1990). Once activated, GR exert effects through both genomic and non-genomic mechanism, both of which are relevant to the notion that Anx-A1 pathway is an important mediator of the anti-inflammatory effects of GCs (Perretti *et al.*, 2009).

Upon ligand binding, hGRα undergoes a conformational change that results in the dissociation of the hsp90 and hsp56. The receptor translocates to the nucleus and binds to specific sequences of DNA known as glucocorticoid response elements (GRE), to either activate anti-inflammatory genes such as IL-10, Anx-A1 and MAPK or suppress pro-inflammatory transcription factors such as AP-1 and NF-kB (Figure 1.5). This action is regulated by phosphorylation signals on GR, which contains multiple phosphorylation sites in the N-terminal domain (Bodwell *et al.*, 1991; Clark *et al.*, 2003).

Human GR has serine phosphorylation sites on Ser^{203}, Ser^{211} and Ser^{226} and it had been shown that these sites have enhanced phosphorylation in response to GC (Ismaili *et al.*, 2004). Phosphatases such as PP2A and PP5 regulate the phosphorylation of GR and indeed a recent study have shown that the defects in PP2A causes GC insensitivity in severe asthma (Kobayashi *et al.*, 2011).

In addition to this genomic pathway, GC also exert acute non-genomic actions that occur immediately after GR ligation (Song *et al.*, 2006). It has been shown that transgenic mouse expressing mutant form of GR, which does not allow translocation of GR upon ligand binding, was still viable suggesting that there may be a substantial number of key effects that are mediated through such cytosolic-GR actions (Reichardt *et al.*, 1999).

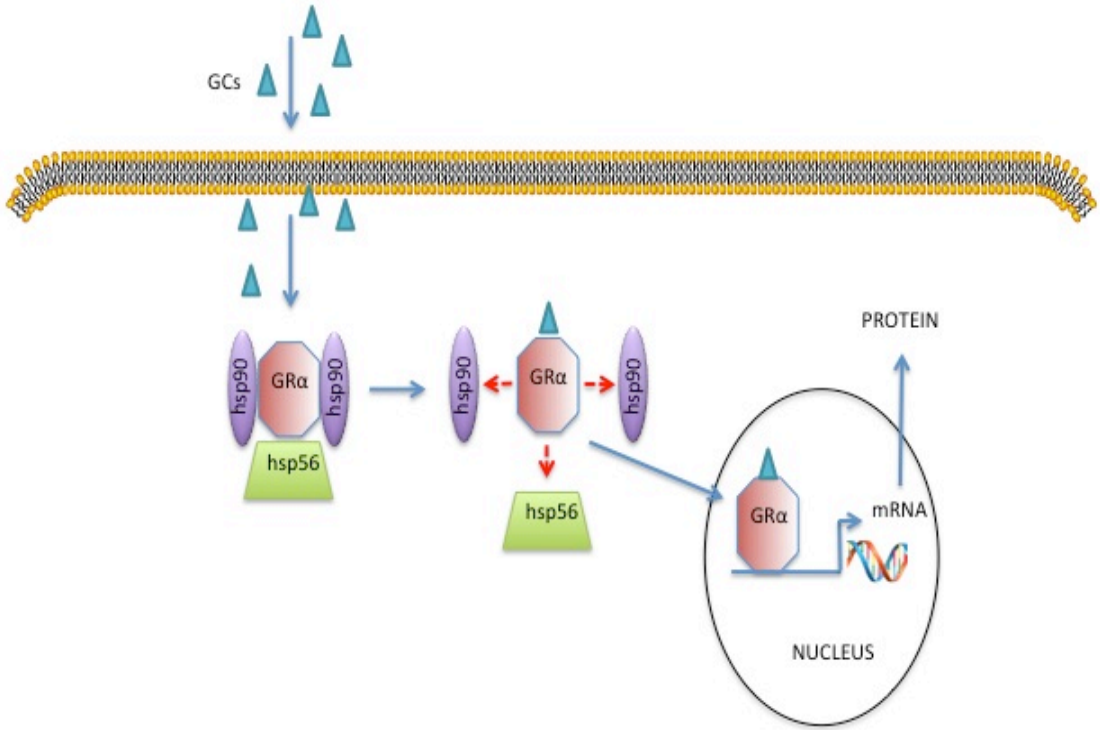


Figure 1.5: Schematic diagram of GR activation and genomic actions.

The inactive GR is localised in the cytoplasm with multiple complex of heat shock proteins (hsp). Upon binding with GCs, the GR undergoes a conformational change, becomes phosphorylated and then dissociates from the hsp complex to translocate to the nucleus. The GR regulates and modulates the inflammatory genes in the nucleus by binding to 'positive' or 'negative' GREs.

1.4 MAST CELLS

1.4.1 Introduction and overview

Paul Ehrlich made the discovery of mast cells in 1878 and first described them on his doctoral thesis. He named them '*mastzellen*', which in German '*mast*' means fattening, as he believed that these cells had nutritional functions because of the granules (Beaven, 2009). Mast cells have long fascinated researchers, and part of this attraction seems to be derived from the lack of consensus of their exact biological functions.

Mast cells are derived from $CD13^+$ $CD34^+$ $CD117^+$ haematopoetic progenitors in the bone marrow (Metcalfe *et al.*, 1997). However mast cells do not circulate in a mature form, instead these cells differentiate and mature in the tissues in which they ultimately reside (Kitamura, 1989; Kawakami *et al.*, 2002). Mast cells are well distributed throughout vascularised tissues, especially near surfaces in contact with the external environment such as the skin, airways and gastrointestinal tract (Metcalfe *et al.*, 1997). Mast cells are similar to monocytes and macrophages, in that they are long-lived cells. Following appropriate stimulation mast cells re-enter the cell cycle and proliferate.

Mast cells have been well characterised as initiators of IgE-dependent allergic diseases but recently, they have also been shown to actively participate in innate and adaptive immune responses to infections and inflammatory autoimmune diseases (Galli *et al.*, 1999; Abraham *et al.*, 2010).

Mast cells have also been reported to participate in inflammatory responses in developing tumors by either enhancing or retarding tumor growth depending on the type of cancer (Galinsky *et al.*, 2008; Ribatti *et al.*, 2012). Other non-immunological roles for mast cells include promotion of angiogenesis, tissue remodelling and wound healing (Noli *et al.*, 2001; Ribatti *et al.*, 2011).

Mast cell degranulation produces a range of characteristics symptoms, which are instantaneous and conspicuous. These includes a string of events including vasodilatation, oedema, muscle contraction, coughing, sneezing, itching, vomiting, diarrhoea and bronchoconstriction (Kaur *et al.*, 2013). Histamine and PGD_2 release are largely responsible for the typical allergic inflammatory symptoms, however PGD_2 also inhibits platelet aggregation and promotes neutrophil infiltration (Schulman *et al.*, 1983; Kovarova *et al.*, 2004).

Mast cells possess a phenotypic 'plasticity', which is characterised by their survival, proliferation, and susceptibility to activation by various stimuli. The ability of these cells to store or produce various secreted mediators and the magnitude of inflammatory responses produced upon activation are 'fine-tuned' by differential expression of receptors and granule constituents (Galli *et al.*, 2005a). Precisely how the mast cells adapt in different immunological or pathological settings remains a matter for debate. However it is now evident that the interaction between surface receptors and the alternative or complementary intracellular signalling pathways profoundly determines the

relative composition and amount of mediators released from activated mast cells (Gilfillan *et al.*, 2011).

Although the activation of mast cells due to the endogenous and exogenous stimuli is a protective mechanism, it could be detrimental in an allergic individual. Pioneering work from Galli's laboratory have highlighted the immunomodulatory role of mast cells and these cells have earned the sobriquet of 'double-edged sword' because of their ability to possess both negative, as well as positive, immunomodulatory roles (Galli *et al.*, 2008).

1.4.2 Mediators released and generated by mast cells

The acute reactions that occur during mast cell activation are triggered by the constitutive release of granules from mast cells and the generation of lipid derived mediators. Histamine is the prevalent granule mediator that initiates the acute reactions due to mast cell activation even though mast cell proteases, such as tryptase, chymase and carboxypeptidase constitute approximately 30-50% of the total protein content of mast cells. Chronic symptoms associated with mast cells are an outcome of the delayed generation of chemokines, cytokines and growth factors (Iwaki *et al.*, 2005b). The process of mast cell degranulation occurs within seconds of mast cell activation and the initial rapid phase is essentially complete within 5-10 min. The late phase reaction develops 2-6 h after exposure of allergen reflecting

on the local recruitment and activation of Th_2 cells, basophils, leukocytes and mast cells.

1.4.2.1 Preformed mediators in granules

Mast cells generate and release a group of pre-formed mediators, such as biogenic amines, lysosomal enzymes, proteoglycans and proteases (Figure 1.3). These mediators are heterogenous, pleiotropic and redundant in that each mediator may have more than one function and the biological effects initiated by the mediators may overlap between one another (Metcalfe *et al.*, 1997).

1.4.2.1.1 Biogenic amines

The principal biogenic amines found in the mast cells are histamine, serotonin and dopamine.

I) Histamine

Histamine was first discovered to be present in the tissue mast cells by Riley and West (RILEY *et al.*, 1952). Histamine is sequestered in mast cell granules by proteoglycans such as heparin and chondroitin E. Storage of histamine is not exclusive to mast cells and basophils as it has been previously demonstrated that histamine is present in a variety of cell types that expresses histidine carboxylase (HDC) such as gastric cells, histaminergic nerve cells, lymphocytes, monocytes, platelets, dendritic cells

and neutrophils (MacGlashan, 2003; Jutel *et al.*, 2005). Histamine modulates cellular activity in many tissues including the dermis, small intestine, stomach, lung and brain. In the mast cells, the classical pathway for histamine release is in response to antigen exposure whereby the surface Fc receptor would bind and crosslink with IgE, resulting in the liberation of large amounts of histamine (10^{-5} to 10^{-3} $molL^{-1}$) during the early allergic response (Jones *et al.*, 2011).

Histamine exerts its biological effects by binding to histamine receptors, which are found in many tissues (Lippert *et al.*, 2004). There are four specific histamine receptors (H_1, H_2, H_3 and H_4) and the effects produced by histamine expression depend on the subtype of receptor involved. All histamine receptors are members of the G-protein coupled receptors (GPCR) family and are coupled to a specific G-protein, resulting in the activation or inhibition of specific pathway (Akdis *et al.*, 2006).

The role of the H_1 receptor is well characterized within the context of allergic inflammation. Liganding of this receptor is followed by activation of the inositol-1,4,5-pathway which leads to the formation of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), hence mobilization of intracellular calcium. This causes the vascular endothelium to release nitric oxide, which in turn results in vasodilatation, erythema, increased vascular permeability and edema (Li *et al.*, 2003). The activation of the H_1 receptor also causes stimulation of sensory nerve endings and leads to the itching sensation of the

mucosa and dermis (Schmelz *et al.*, 1997). H_1 receptor occupancy in bronchial smooth muscles leads to bronchoconstriction.

The H_2 receptor is expressed on gastric mucosal cells, vascular smooth muscle, brain, adipocytes and immune cells. Activation of this receptor results in relaxation of smooth muscle in the airway and vasculature (Akdis *et al.*, 2006). This receptor has also been reported to be involved in the regulation of cell proliferation, particularly in carcinoma cells. However the most studied physiological response of H_2 receptor is gastric acid secretion, and this receptor is involved in the regulation of gastric motility and intestinal secretion of bicarbonate through the inhibition of prostaglandin E_2 (PGE_2). Whereas in the cardiovascular system, activation of this receptor causes decreased chronotropic and ionotropic effects (Del Valle *et al.*, 1997). The H_2 receptor also regulates the immunoregulatory function of histamine as it has been shown that the activation of this receptor has inhibitory actions on the chemotaxis of neutrophils, basophils and eosinophils (Jones *et al.*, 2011). Interestingly, it has been demostrated that H_2 receptor may have immunosuppressive effects in the control of inflammation induced by histamine (Jutel *et al.*, 2001) because Th_1 and Th_2 cells are negatively regulated by this receptor (Seligmann *et al.*, 1983).

The H_3 receptor was discovered in 1983 (MacGlashan, 2003) and cloned in 1999, and was found to be expressed not only on histaminergic neurons but also on eosinophils, monocytes and dendritic cells (Lovenberg *et al.*, 1999). This receptor regulates the neuronal-mast cell feedback loop by

mediating the synthesis of histamine and inhibition of its release from histaminergic neurons. The H_3 receptor has been implicated in regulation of sleep-wake cycles (Griebel *et al.*, 2012), obesity (Hancock, 2003), cognition (Fox *et al.*, 2003) and allergic rhinitis (Suzuki *et al.*, 2008). Exploitation of this receptor could lead to a potential therapeutics in the field of neuro-inflammatory research.

The H_4 receptor is the latest addition to the histamine receptor family. It was identified in 1994 and cloned in 2000 (Nijmeijer *et al.*, 2012). The H_4 receptor shares 35% amino acid homology to H_3 receptor. This receptor is expressed on eosinophils, basophils, T-cells, monocytes, mast cells, spleen, thymus, small intestine, colon and heart (Akdis *et al.*, 2003; Akdis *et al.*, 2006). The physiological functions of H_4 receptor demonstrated so far are on eosinophils (Ling *et al.*, 2004a), mast cells (Hofstra *et al.*, 2003) and monocyte-derived dendritic cell chemotaxis (Gutzmer *et al.*, 2005), regulation of T-cells or dendritic cell functions and mast cell dependent neutrophil migration (Takeshita *et al.*, 2003). Activation of this receptor leads to the calcium ion mobilization in mast cells and inhibition of cAMP increase (Hofstra *et al.*, 2003). A separate study has shown that activation of the H_4 receptor induces mitogen-activated protein kinase (MAPK) phosphorylation, which was inhibited by pertussis toxin (Morse *et al.*, 2001). The H_4 receptor may play a role in pruritis as it was demonstrated that H_4 receptor knock-out mice experienced significantly lesser pruritis after exposure to exogenous histamine as compared to the wild type mice, and that treatment with a

selective H_4 receptor inverse agonist significantly reduced the itch (Dunford *et al.*, 2007).

An elegant study by Thurmond *et al* demonstrated the importance of H_4 receptor in mast cell chemotaxis (Thurmond *et al.*, 2004). Experimental mice were challenged with aerosolised histamine to mimic the allergic response in the lungs and a redistribution of mast cells in the tracheal epithelium was observed. This effect was blocked by systemic administration of a known selective H_4 inverse agonist.

II) Serotonin

Serotonin is a bioactive mediator associated with many physiological processes including immunomodulation (Arzt *et al.*, 1988), cell growth and development, mast cell adhesion and chemotaxis (Kushnir-Sukhov *et al.*, 2006), tumor growth (Siddiqui *et al.*, 2005), and tissue regeneration (Lesurtel *et al.*, 2006). Serotonin binds to the 5-hydroxytryptamine (HT) receptor, which mediates both excitatory and inhibitory neurotransmission. Serotonin is synthesized by the hydroxylation of tryptophan, in a reaction catalysed by tryptophan hydroxylase (TPH). Several functions of serotonin have been elucidated in animal strains lacking TPH1 and TPH2 (Alenina *et al.*, 2009), which are the two crucial isoforms of tryptophan hydroxylase (TPH). TPH1 appears to be the more predominant isoform in mast cells and its levels correlates well with the degree of mast cell maturation (Ringvall *et al.*, 2008). The notion that serotonin was mainly found in the granules of rodent mast cell

and that human mast cells lacked stored serotonin (SJOERDSMA *et al.*, 1957) has recently been challenged by Metcalfe and colleagues, who demonstrated that human peripheral blood-derived mast cells contains serotonin and plasma serotonin levels were elevated in patients suffering from mastocytosis (Kushnir-Sukhov *et al.*, 2007).

III) Dopamine

To date there has been very little evidence that suggest that dopamine is synthesized and stored in mast cells. However it has been shown that mast cell activation results in the depletion of cell-associated dopamine (Lesurtel *et al.*, 2006), although mRNAs coding for the enzymes that catalyses the formation of dopamine has not been identified in mast cells (Lundequist *et al.*, 2011).

Preformed mediators stored in MC secretory granules
1. Biogenic amines • Histamine • Serotonin • Dopamine
2. Lysosomal enzymes • β-hexosaminidase • β-glucuronidase • β-D-galactosidase • Arylsulfatase A • Cathepsin C • Cathepsin B • Cathepsin L • Cathepsin D • Cathepsin E
3. Proteoglycans • Serglycin (with heparin and/or chondritin sulphate GAG chains)
4. Proteases • Chymase • Tryptase • MC-CPA • Cathepsin G • Granzyme B • MMP-9 • Renin

Table 1.3: Main preformed mediators, which are stored in the secretory granules of mast cells.

1.4.2.1.2 Lysosomal enzymes

A variety of enzymes have been localized to mast cell granules as evidenced by histochemical techniques and subcellular fractionation. Mast cell secretory granules are quite similar to lysosomes in terms of their acidic pH and membrane components such as vesicle associated membrane protein (VAMP-8) and soluble NSF attachment protein receptor (SNARE) proteins; hence these granules are often referred to as 'secretory lysosomes' (Puri *et al.*, 2008; Tiwari *et al.*, 2008). The biological functions of lysosomal enzymes in mast cells has not been well elucidated yet, however these enzymes might have a role not only in the normal intracellular turnover of the cells but also in the extracellular sequence of mast cell degranulation (Lundequist *et al.*, 2011). The most ubiquitous lysosomal enzyme present in mast cells is β-hexosaminidase, thus its release is frequently used as means of quantifying the extent of mast cell degranulation. Other lysosomal enzymes present in mast cells are a number of saccharide-degrading enzymes, including β-glucuronidase, β-galactosidase and arylsulfatase A (Schwartz *et al.*, 1981).

Recent studies have also shown that mast cell granules contain lysosomal proteases such as cysteine cathepsins (cathepsin C, B and L) (Dragonetti *et al.*, 2000; Wolters *et al.*, 2000) and aspartic acid proteases (cathepsin D and E) (Dragonetti *et al.*, 2000; Henningsson *et al.*, 2005). IgE-mediated mast cell degranulation was shown to induce the release of

cathepsin B, D, and L (Dragonetti *et al.*, 2000), thus further indicating their presence in the secretory granules.

Traditionally, lysosomal enzymes have been considered to be mainly active within the acidic environment of lysosomes and then to become rapidly inactivated after exposure to extracellular pH. However, recent studies have revealed that several lysosomal cathepsins possess significant enzymatic activity even after cellular release (Turk *et al.*, 2001). Hence, the extracellular functions of lysosomal enzymes should not be disregarded.

1.4.2.1.3 Proteoglycans

The major constituents of mast cell granules are serglycin proteoglycans, which contain a protein core that is attached to heavily sulfated and negatively charged glycosaminoglycan (GAG) side chains. The expression of serglycin varies depending on cell types. In the connective tissue-type mast cells of rodents, highly sulfated GAGs of heparin are more prevalent, whereas in mucosal mast cells, chondroitin sulfate is the predominant GAG component of serglycin (Yurt *et al.*, 1977; Enerbäck *et al.*, 1985). However, in human mast cells, serglycin contains both heparin and chondroitin sulfate in a ratio of 2:1 (Metcalfe *et al.*, 1979; Thompson *et al.*, 1988).

A unique characteristic of mast cell serglycin is that the GAG chains have a high level of sulfation as compared to serglycins from other cell types.

This feature enables them to form tight electrostatic interactions with other basic compounds that co-exist in the granules. Even though the reason for this is not completely understood, recent research has shown that mast cell maturation correlates positively with the expression of chondroitin sulfate and heparin sulfotransferases (Duelli *et al.*, 2009).

The high degree of overall sulfation by mast cell serglycin results in an elevated total anionic charge density, which forms the basis for the strong staining with cationic dyes (Braga *et al.*, 2007). Histologically, mast cells are easily distinguished by their strong granular staining with various cationic dyes such as Toluidine blue, Alcian blue, Berberine sulphate and May Grünwald/Giemsa. The staining with these dyes are explained by their strong binding to serglycin present in mast cell granules as it had been previously demonstrated that the mast cells from serglycin null mice completely lacked metachromatic staining (Abrink *et al.*, 2004).

1.4.2.1.4 Proteases

Mast cell proteases accounts for more than 25% of the total protein content of mast cells, thus representing the major group of mediators released during mast cells degranulation (Schwartz *et al.*, 1987). Even though a myriad of potential functions of mast cell proteases have been previously outlined, it is only recently that the in-vivo functions of these proteases have

been clarified by experimental approaches using mast cell protease deficient mice (Pejler *et al.*, 2007).

The proteases expressed specifically by mast cells include tryptase, chymase and carboxypeptidase A. Indeed, mast cells also express a number of additional non mast cell-specific proteases such as lysosomal cathepsins, granzyme B (Pardo *et al.*, 2007), neurolysin (Pejler *et al.*, 2007), cathepsin G (Schechter *et al.*, 1990; Schechter *et al.*, 1994), matrix metalloprotease 9 (MMP 9) (Baram *et al.*, 2001) and renin (Silver *et al.*, 2004). It is noteworthy that the specific mast cell protease repertoire differs between mast cell subsets depending largely on phenotype and distribution of mast cells within the tissue.

In humans, mast cells are classified according to their protease content, with the MC_T subclass expressing tryptase only and the MC_{TC} subclass expressing tryptase, chymase and carboxypeptidase A (Irani *et al.*, 1986). Past research has shown that the mast cells derived from the cord blood exhibit a phenotype in common with MC_{TC} subclass of human mast cells (Nilsson *et al.*, 1996). However in mouse, mast cells are sub-divided into the connective tissue mast cell (CTMC) and mucosal mast cell (MMC) subtypes. Storage of mast cell proteases are highly efficient, made possible by their tight packaging in complexes with serglycin proteoglycan (Pejler *et al.*, 2009). In support of this notion, the deletion of the serglycin gene was shown to almost completely eradicated the ability of mast cells to store mast cell proteases (Abrink *et al.*, 2004).

Recent studies with the mast cell-protease null mouse strains have highlighted the important roles of these enzymes. There are two main enzymatically active tryptases, α- and β- tryptase, that are released during mast cell degranulation. The study of the biological functions of tryptase was made possible through the generation of mouse strains deficient in mMCP-6, the murine tryptase that shares a close homology to the human β- tryptase (Stevens *et al.*, 2007). Past studies have demonstrated that tryptase contributes to the elimination of parasitic infestations by the innate immune response although it is not essential (Shin *et al.*, 2008). Although mast cells play an important role in the defence against bacterial infections, the mechanisms by which this occurs have not been convincingly elucidated. Given the ability of mMCP-6 to recruit neutrophils, Thakurdass *et al* reported that indeed, mMCP-6 contributes to the defense againts intraperitoneal bacterial infection by inducing neutrophil infiltration, which was only evident during the early phases of infection, in line with the postulated primary role of mast cells as the first-line of defence against bacterial invasion (Thakurdas *et al.*, 2007). An elegant study was conducted by McNeil *et al* to understand the role of mast cells in experimentally induced arthritis (McNeil *et al.*, 2008). The authors have shown that mMCP-6$^{-/-}$ mice developed less severe joint inflammation as compared to the wild type mice when arthritis was passively induced by methyl-bovine serum albumin/interleukin 1β (IL-1β). A similar observation was noted in the K/BxN model of arthritis, whereby mMCP-6$^{-/-}$ mice developed lower clinical scores, inflammation and bone/cartilage

erosions as compared to the wild type strain. Even though these studies indicate that mast cell tryptase plays an important role in the development of arthritis, the involvement of tryptase in other models of arthritis such as by immunization of collagen II is yet to be investigated.

Chymase, which is fairly selectively expressed by mast cells, has been associated in various pathological conditions (Caughey, 2007). To further investigate the role of chymase in human diseases, mouse strains deficient in mMCP-1, mMCP-4 and mMCP-5 genes have been generated. However, it is extremely important to determine which of the murine chymase enzymes shares the most sequence similarity with the human chymase. Even though mMCP-5 is the closest homolog to the human chymase, cleavage specificity investigations have revealed that this enzyme has elastase, rather than chymotrypsin-like substrate specificity. Hence mMCP-5 is not considered to be a functional homolog to human chymase (Kunori *et al.*, 2002). mMCP-2 strains have been reported to have different homology to the human chymase (Andersson *et al.*, 2008b). Whereas the mMCP-4 gene has similar tissue redistribution, substrate cleavage specificity and serglycin-binding properties compared to human chymase (Andersson *et al.*, 2008a; Andersson *et al.*, 2009). Collectively, these facts indicate that mMCP-4 is the closest functional homology to the human chymase and thus the mMCP-4 null mice would be a more relevant tool to elucidate the function of human chymase. Studies have shown that the phenotype of mMCP-4 null strain leads to less severe collagen induced arthritis (Magnusson *et al.*, 2009), increased susceptibility to

allergic airway inflammation (Waern *et al.*, 2009), decreased development of abdominal aortic aneurysm (Sun *et al.*, 2009), increased tissue accumulation of extracellular matrix (ECM) due to the defective fibronectin (Tchougounova *et al.*, 2003; Tchougounova *et al.*, 2005) and reduced basal intestinal permeability (Groschwitz *et al.*, 2009). Taken together, it cannot be excluded that other murine chymases might mimic the corresponding functions of chymase in human.

Another mast cell protease is carboxypeptidase A (MC-CPA), which is a highly conserved secretory granule. The tissue location and other properties of MC-CPA enzyme between human and mouse are similar (Feyerabend *et al.*, 2005), thus it is probable that functions of this gene determined in the MC-CPA null mouse would correspond to that of the human counterpart. Previous studies using MC-CPA knock-in strains and shRNA approach have shown that MC-CPA may play a role in the clearance of endothelin-1, an endogenous peptide released during bacterial infection (Maurer *et al.*, 2004; Schneider *et al.*, 2007) and degradation of certain snake venom toxins (Metz *et al.*, 2006).

1.4.2.2 Eicosanoids and reactive oxygen species

Mast cell activation also provokes acute *de novo* synthesis and release of lipid mediators such as eicosanoids. Upon stimulation with antigen, complement or other stimuli, mast cells release eicosanoids, growth factors and various cytokines and chemokines (Table 1.4). Among the known lipid mediators of mast cell, eicosanoid plays a prominent role.

It has been established that mast cells release eicosanoids such as prostaglandin (PG) D_2, leukotriene (LT) B_4, and LTC_4 (MacGlashan *et al.*, 1982; Peters *et al.*, 1984; Murray *et al.*, 1986) in response to appropriate stimuli. These eicosanoids are derived from arachidonic acid (AA), a 20-carbon fatty acid released from the nuclear membrane phospholipids by cytosolic phospholipase A ($cPLA_2$) (Clark *et al.*, 1995). This functional activation requires intracellular calcium flux and phosphorylation of mitogen-activated protein kinases (MAPKs) (Berenbaum *et al.*, 2003). When released each eicosanoid acts through specific G-protein coupled receptors (GPCRs) which in turn, regulate certain functions of mast cells through paracrine, autocrine and intracrine mechanisms (Boyce, 2007). These diverse lipid mediators are capable of initiating, amplifying or dampening the inflammatory responses. The magnitude, duration and nature of the subsequent immune responses are also regulated by eicosanoids released from mast cells.

PGD_2 is the major cyclooxygenase (COX) metabolite of arachidonic acid that is released upon stimulation of mast cells. COX-1 and COX-2 are

both expressed in mast cells and the latter can be upregulated by interleukin (IL)-1β, suggesting a mechanism that can augment PGD_2 production under inflammatory conditions.

When atopic human subjects are challenged with specific allergen, the level of PGD_2 is increased in the bronchoalveolar lavage (BAL) fluid (Wenzel *et al.*, 1989). The amounts of 9α, 11β-PGF_2 which is the primary PGD_2 metabolite and which shares the biological activity of its parent compound (Beasley *et al.*, 1987) is also elevated in both serum and urine of patients with allergen-induced activation of mast cells (Bochenek *et al.*, 2004). PGD_2 was reported to induce bronchoconstriction at levels 10-fold lower than histamine when administered by inhalation to patients with asthma (Hardy *et al.*, 1984) and to cause wheal-and-flare responses in the skin of atopic individuals indicating that PGD_2 also has a role at the microvasculature (Soter *et al.*, 1983). To further elucidate the role of PGD_2, animal models lacking DP1 and DP2 receptors for PGD_2 have been generated. The human DP1 receptor mRNA is highly expressed in retina and small intestine (Boie *et al.*, 1995) whereas the DP2 receptor is expressed almost by all tissues, however it is most abundant in heart, brain, stomach, adrenal gland, liver, small intestine, thymus and placenta (Sawyer *et al.*, 2002).

Newly synthesised mast derived mediators
1. Lipid-derived • Leukotrine B4 (LTB_4) • Leukotrine C4 (LTC_4) • Prostaglandin D_2 (PGD_2)
2. Cytokines • Tumor necrosis factor (TNF)-α • Ganulocyte macrophage colony stimulating factor (GM-CSF) • IL-3 • IL-4 • IL-5 • IL-6 • IL-10 • IL-13
3. Chemokines • CCL2 • CCL3 • CCL5 • CCL8
4. Growth factors • SCF • FGF • VEGF • Angiogenin

Table 1.4: *De novo* synthesized mast cell mediators.

1.4.2.3 Cytokines and chemokines

It is clear from the seminal observations of several investigators, that cytokines are generated and stored within the secretory granules of mast cells (Burd *et al.*, 1989; Plaut *et al.*, 1989; Gordon *et al.*, 1990a). Mast cell degranulation augments gene expression, which leads to the generation of a range of cytokines including tumor necrosis factor- α (TNF-α) (Gordon *et al.*, 1990b), granulocyte macrophage-colony stimulating factor (GM-CSF), IL-3, IL-4, IL-5, IL-6, IL-10 and IL-13 (Renauld, 2001).

Chemokines such as CCL2, CCL3, CCL5 and CXCL8 and growth factors such as stem cell factor (SCF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and angiogenin are released by mast cells during activation. There has also been increasing appreciation that mast cell activation can be regulated by these cytokines, chemokines and growth factors (Katsanos *et al.*, 2008).

Types of growth factors released upon mast cell degranulation include TGF-β (Lindstedt *et al.*, 2001), nerve growth factor (NGF) (Leon *et al.*, 1994), SCF (Zhang *et al.*, 1998) and IL-4 (Horsmanheimo *et al.*, 1994) from various types of mast cells.

1.4.3 Mast cells activating ligands, receptors and signalling

Antigen-dependent mast-cell activation is regulated by a complex series of intracellular signalling processes that is initiated following FcεRI aggregation. Although the immediate receptor-upstream signalling events seem to be common for mast cell degranulation, the receptor-downstream signalling events must converge to regulate the different mechanisms by which these mediators are released (Figure 1.7).

1.4.3.1 High affinity receptor for IgE, FcεR1

Mast cells play a part in a diverse array of physiological and pathological processes by responding to many different stimuli, such as stem cell factor (SCF), IL-3, lipopolysaccharide (LPS), certain products of complement activation and neuropeptides as a result of activation of their various receptors (Kitamura, 1989; Galli *et al.*, 1994; Metcalfe *et al.*, 1997). Nonetheless, the most researched mechanism by which mast cells execute immunological functions is through the antigen- and Ig-E-dependent aggregation of high affinity IgE receptor, FcεR1 (Galli *et al.*, 2005a). Activation of this receptor leads to the downstream events that result in the generation or release of mast cell mediators (Gilfillan *et al.*, 2006) and mast cell chemotaxis (Kuehn *et al.*, 2010). However, it has been previously noted that FcεR1 also influences mast cell biology indirectly as monomeric IgE

promotes mast cell survival (Asai *et al.*, 2001; Kalesnikoff *et al.*, 2001), induces cell migration (Kitaura *et al.*, 2005) as well as the generation of cytokines (Matsuda *et al.*, 2005).

FcεR1 (Figure 1.6) is a heterotetrametric receptor, comprising of IgE-binding α subunit, four transmembrane-spanning β subunits, and two identical disulphide linked γ subunits (Turner *et al.*, 1999). Monomeric IgE binds to FcεR1 with very high affinity, thus this complex is retained for long duration, thereby setting the platform for immediate allergic reactions or inflammatory response upon contact with antigen. The β subunits amplify the signalling events induced by IgE- and Ag complex and the γ subunits are essential for instigating the downstream signalling events because they contain one immunoreceptor tyrosine-based activation motif (ITAM) (Turner *et al.*, 1999; Nadler *et al.*, 2000).

Coordinated orchestration of sequential, parallel and interacting signalling pathways are required to release all the major inflammatory mediators from mast cells. Indeed, it is the divergence of these pathways that allows chemotaxis or the release of one type of mediator in the absence of the others. Even though the pathways governing the mast cell activation are complex, they can be broadly divided into the following categories.

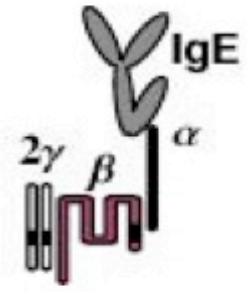


Figure 1.6: The subunits of FcεR1.

FcεR1 comprises of one α subunit, four β subunits and two γ subunits. This receptor binds to IgE with high affinity.

I) FcεR1 β and γ phosphorylation leading to Syk recruitment/activation

Signalling events following FcεR1 aggregation require the initial phosphorylation of specific tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs) contained within the β and γ chains of the FcεR1. This leads to the recruitment of the proto-oncogene tyrosine-protein kinase Src family kinases, Lyn (Eiseman et al., 1992; Vonakis et al., 2005) and Fyn (Parravicini et al., 2002) to the β chain and spleen tyrosine kinase (Syk) to the γ chain homodimer (Shiue et al., 1995). Even though it has been suggested that the main kinase responsible for this initial phosphorylation is Lyn, there is convincing evidence suggesting that other kinases may alternatively regulate this compulsory event (Odom et al., 2004). However, the recruitment of Syk to the phosphorylated γ chains and the sequential downstream activation is a mandatory event for mast cell activation.

II) The LAT/PLCγ-Calcium/PKC Axis

The receptor-signalling complex is synchronised through a series of constitutive and inducible protein-protein and protein-lipid interactions through a network of transmembrane and cytosolic adapter proteins (Iwaki et al., 2005a). These interactions occur in glycolipids enriched localised regions, known as lipid rafts (Dráber et al., 2002; Sengupta et al., 2007). The ability of surface receptors to preferentially engage specific adaptor molecules to recruit a particular signalling element is reflected by the underlying capacity of these receptors to independently regulate the release of specific mast cell mediators.

The major substrate for Syk is the transmembrane adaptor molecule linker of activated T cells (LAT), but it is also the central regulator of the downstream events necessary for mast cell activation upon FcεR1 aggregation (Wonerow *et al.*, 2001). Syk and potentially other kinases phosphorylate LAT and the latter recruits phospholipase γ1 (PLCγ1) and the cytosolic adaptor molecules SLP76 and indirectly GRB2-related adapter protein (Gads) (Silverman *et al.*, 2006). This interaction is considered vital, as it was previously demonstrated that there was a pronounced attenuation of the ability of antigens to elicit calcium response, mast cell degranulation and generation of cytokines in the mast cells derived from $lat^{-/-}$ (Saitoh *et al.*, 2000), $slp^{-/-}$ (Pivniouk *et al.*, 1999) and $gads^{-/-}$ (Yamasaki *et al.*, 2008) mice.

PLCγ exists as two isoforms, which is PLCγ1 and PLCγ2. Both of these isoforms are expressed in mast cells and activated following FcεR1

aggregation, even though PLCγ1 is the more dominant form in human (Wilde *et al.*, 2001). PLCγ activation leads to the hydrolysis of the membrane-asscociated phospholipid, phosphoinositide 4,5 biphosphate (PIP_2) thus liberating inositol triphosphate (IP_3) and diacylglycerol (DAG) (Choi *et al.*, 2007). This events leads to the release of calcium from endoplasmic reticulum, and activates PKC isoforms, which are crucial for mast cell activation (Ozawa *et al.*, 1993; Ma *et al.*, 2009). The extracellular calcium influx is essential for a variety of downstream signalling processes not only triggering mast cell degranulation (Ashmole *et al.*, 2013) but also for the generation of other mediators and for cell chemotaxis (Ma *et al.*, 2011). Calcium is also critical for the activation of PLA_2, which is essential for the generation of arachidonic acid leading to eicosanoid generation and cytokine production (Di Capite *et al.*, 2011).

III) The PI3K/BTK Axis

The phosphatidylinositide 3-kinase/Brutons' tyrosine kinase (PI3K/BTK) axis governs the maintenance and amplification of the signal derived from the activation of PLCγ (Iwaki *et al.*, 2005b). Even though the initial phase of calcium signal mediated by PLCγ is unaltered by the inhibition of PI3K, the maintenance of this signal has significantly reduced following the depletion of PI3K (Ali *et al.*, 2004).

PI3K is recruited to the signalling complex following the Fyn-dependent binding of PI3K to Gab2 (Yu *et al.*, 2006). The membrane localisation of the

specific signalling molecule is promoted through the generation of phosphatidylinositol 3, 4, 5 triphosphate (PIP_3), which bind to the pleckstrin homology (PH)-domains (Kim *et al.*, 2008). Btk is activated once it is recruited to the cell membrane, and this activation further increases PLCγ activity and reinforces its capacity to promote calcium signal (Kuehn *et al.*, 2010).

The multifaceted role of PI3K is revealed through experiments utilising mouse mast cells expressing catalytically inactive PI3K or using PI3K inhibitors. PI3K regulates not only mast cell degranulation but also the generation of reactive oxygen species (ROS), eicosanoid and cytokine production, cell growth and survival, cell adhesion and chemotaxis (Kim *et al.*, 2008; Kuehn *et al.*, 2008).

IV) MAP kinase pathways and transcriptional regulation.

The activation of the mitogen-activated protein (MAP) kinase pathways is crucial for receptor-mediated generation and release of cytokines, chemokines, growth factors and other proteins in mast cells. There are three major MAP kinase pathways, that is the extracellular signal regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38, which regulates the activity of numerous transcription factors important for cytokine production and the activity of PLA_2 (Gilfillan *et al.*, 2009).

All these MAP kinases pathways are activated through the Ras-Raf pathway in antigen stimulated cells. The Ras-Raf-MAP kinase pathway and the PI3K-regulated signalling network contribute to the activation of the

transcriptional factors, which includes fos and c-jun (Turner *et al.*, 1997; Novotny *et al.*, 1998). These signalling cascades are vital to the generation and release of cytokines and chemokines (Gilfillan *et al.*, 2006).

Both PKC and calcium, which are regulated by the LAT/PLCγ_1/calcium/PKC axis also contributes to transcriptional regulation independent of MAP kinases and PI3K (Gilfillan *et al.*, 2006). The signal transduction events induced by these stimuli can interact with those initiated through FcεR1 to enhance cytokine production without altering degranulation (Qiao *et al.*, 2006; Andrade *et al.*, 2011).

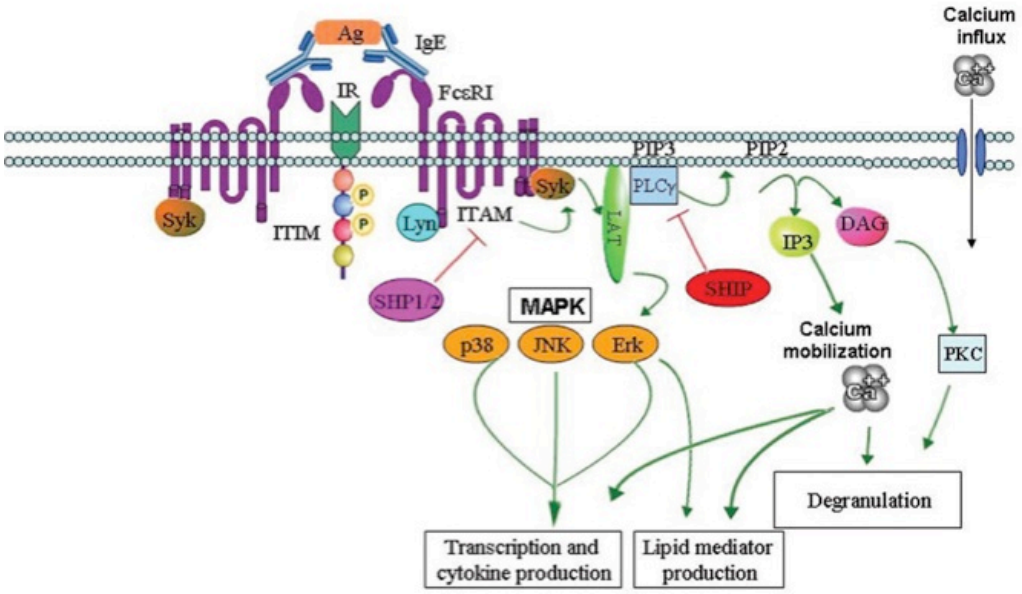


Figure 1.7: Schematic representation of FcεRI-mediated mast cell activation.

Mast cell FcεRI aggregation by receptor-bound IgE with an antigen leads to the phosphorylation of the receptors ITAMS by kinase Lyn. Sky is recruited to the complex and phosphorylates LAT, which activates PLCγ to degrade PIP2 to IP_3 and DAG. IP_3 induces intracellular Ca^{2+} mobilisation followed by extracellular influx whilst DAG activates PKC isoforms. LAT also serves as a docking site for adapter molecules, which leads to a downstream activation of MAP kinases (JNK, p38 and ERK). These sequential events lead to mast cell degranulation, cytokine and lipid mediator production (Karra *et al.*, 2011).

1.4.3.2 C-kit receptor

C-kit receptor is a growth factor receptor. Activation of this receptor is not only essential for the mast cell growth, differentiation and survival, but it is also extremely important for mast cell migration or homing through chemotaxis. The c-kit receptor consists of a single chain receptor with five extracellular immunoglobulin-like domains and a tyrosine kinase catalytic domain in the cytosolic tail. Binding of the c-kit receptor and its specific ligand, stem cell factor (SCF) triggers the catalytic activity and downstream signalling cascade. SCF is found in two major forms, the soluble form and the membrane-bound form. This ligand is primarily expressed by fibroblast but is also generated by mast cells and other tissues.

Under experimental conditions, SCF itself can regulate mast cell degranulation and cytokine production (Bischoff *et al.*, 1992; Hundley *et al.*, 2004) However, some previous studies have shown that the signals generated by the activated c-kit receptors are insufficient to induce degranulation alone and need to be accompanied by other mast cell stimulants to trigger this effect. Nonetheless, c-kit receptors could induce the generation of cytokines without the presence other stimulants (Hundley *et al.*, 2004; Tkaczyk *et al.*, 2004). Additionally, SCF and monomeric IgE can promote mast cell degranulation synergistically, although neither can induce degranulation on their own (Cruse et al., 2005).

The signalling pathways elicited by c-kit receptor ligation does overlap with those induced by FcεR1, although c-kit is unable to compensate for the critical signals required to induce mast cell degranulation (Hundley et al., 2004). Even though, c-kit activates Src kinases, PLCγ, PI3K and MAPK cascade (Iwaki et al., 2005b), it is not able to recruit and activate Syk, phosphorylate LAT (Tkaczyk et al., 2004) and activate PKC (Hundley et al., 2004). These absent or weak signals account for the inability of c-kit to produce mast cell degranulation. However, in the presence of FcεR1 aggregation, c-kit potentiates degranulation by other agents and cytokine generation.

1.4.3.3 Other activators with undefined receptors and mechanisms of activation; compound 48/80.

Mast cell 'secretagogues' are a diverse group of cationic compounds known to cause rapid mast cell degranulation, which were used before the mechanism of IgE induced mast cell activation was discovered. Examples of these secretagogues include the polymeric compound 48/80, polyamines such as spermine and spermidine, various kinins, cationic peptide hormones and hymenoptera venom substituents and mast cell degranulating peptides (Mousli *et al.*, 1990a; Mousli *et al.*, 1990b).

Compound 48/80 is a mixed polymer of p-methoxy-N-methyl phenethylamine cross-linked to formaldehyde (Lagunoff *et al.*, 1983) and is

widely used for non-IgE dependent stimulation of mast cells (Metcalfe *et al.*, 1997). Compound 48/80 was first characterised as an agent that released histamine (PATON, 1951) and many studies have used this compound as a mast cell secretagogue or to evoke inflammatory responses in various animal models (Kubes *et al.*, 1996; Schemann *et al.*, 2012).

However, identification of the signaling cascades triggered by compound 48/80 and other classic 'secretagogoues' is vague and incomplete. Several studies have revealed that compound 48/80 acts via the pertussis toxin-sensitive heterotrimeric G_i proteins and triggers the transient production of inositol phosphates and Ca^{2+}-influx (Nakamura et al., 1985), which liberate rapid exocytosis (~ 10 sec) of histamine (Rothschild, 1970), arachidonic acid (McClain et al., 1984) and PGD_2 (van Haaster et al., 1995). In contrast, IgE-dependent exocytosis is relatively slow, extending over a period of ~ 2 min, is strictly dependent on the presence of external Ca^{2+} (Foreman *et al.*, 1972), and is pertussin toxin insensitive. Hence, basic secretagogues appear to induce secretion by a different mechanism to that mediated by IgE.

More recently, literature has suggested a direct interaction of compound 48/80 with the COOH-terminal end of α-subunit of G_{i3} at the plasma membrane (Aridor et al., 1993). This induces signals such as PKC and PI3K activation (Shefler et al., 1998) essential for the activation of PLA_2 with the associated production of arachidonic metabolites such as PGD_2 (Byrne et al., 2007). In addition to degranulation, compound 48/80 induces

the production and release of IL-3, IL-8, TNF-α and GM-CSF (Kulka et al., 2008).

1.5 ALLERGIC INFLAMMATION

Allergy was a term coined by Clemens von Pirquet in 1906, to refer to the abnormal adaptive immune responses involving allergen-specific IgE and T-helper 2 (Th2) cells that recognise allergen-derived antigens (Niederberger, 2009). An allergic reaction could be defined as hypersensitivity of the immune system which is activated due to a complex interplay between inflammatory cells, including mast cells, dendritic cells, basophils and eosinophils (Barnes, 2011b). This reaction is triggered when IgE excessively activates mast cells and basophils (Kay, 2000). During an allergic reaction, IgE bound antigen that activates FcεRi increases calcium permeability of mast cells in a ATP dependent manner releasing pro-inflammatory mediators such as histamine.

The common allergic diseases such as asthma, allergic rhinitis and atopic dermatitis (Broide, 2009; Hamid *et al.*, 2009) share common features of the inflammatory processes. The characteristic of allergic inflammation involves the IgE-dependent activation of mucosal mast cells and the infiltration of eosinophils, which intertwines with the increased $CD4^+$ Th2 lymphocyte activation. However, the clinical differences between these diseases are apparent anatomically and by the interaction between allergic inflammation and structural cells. It is now apparent that the structural cells in

the airways and skin play an essential role in the secretion of inflammatory mediators and in maintaining the chronic allergic inflammation (Barnes, 2011b). The changes in the smooth muscle cells in the lower airways, results in bronchoconstriction and vasodilatation that leads to nasal blockage and activation of keratinocytes in the skin.

Genetic factors play an important role on whether atopy develops, and may exert an influence on the severity of the disease and the extent of the allergic inflammatory response. Allergy frequently starts in early childhood with initial eczema, followed by sequential development of food allergy, allergic rhinitis and asthma (Eder *et al.*, 2006). Environmental factors such as pollens, dust mites, cat and food may influence whether an atopic individual develops a particular allergic disease. Several genes have been identified using the genome-wide association studies (GWAS) (Akhabir *et al.*, 2011), although each identified gene only contributes to a small amount to the observed phenotype (Ober *et al.*, 2006).

Although priority has been given on the inflammatory mechanisms in allergy, there are several endogenous anti-inflammatory mechanisms that could be defective in allergic diseases. One such example is endogenous cortisol, which is an important regulator of the allergic inflammatory response. Exacerbation of asthma at night might be related to the circadian cycle of plasma cortisol and blockade of endogenous cortisol by metyrapone results in an increase in the late response to allergen in the skin (Herrscher *et al.*, 1992).

Even though allergic inflammation is a complex disease involving many cells and mediators, many therapeutic targets have been identified. However, treatment with a broad spectrum of action is needed as targeting individual mediators or cell types are unlikely to produce effective therapies for allergic diseases. Corticosteroids are very effective in treating allergic diseases as they have multiple anti-inflammatory effects, including suppression of inflammatory genes (Barnes, 2006), although it has been reported that inhaled corticosteroid treatment is more effective for asthma patients compared to patients with chronic obstructive pulmonary disease (COPD) (Belvisi, 2004). The recent recognition of epigenetic factors likely to be important in regulating the inflammatory genes involved in allergic diseases might lead new therapeutic targets in the future.

1.5.1 Histamine receptor 1 (H_1) Antagonists

A H_1 antagonist drug is characterised by its ability to block the histamine receptor 1, hence reducing or eliminating the effects mediated by histamine during an allergic response. Only drugs that act by mediating the negative modulation of histamine receptors are termed anti-histamines (Simons *et al.*, 1994). However, the first generation of anti-histamines possesses adverse effects such as sedation, impaired psychomotor ability and excessive mucosal drying (Simons, 1994). Thus the development of second-generation H_1 antagonist drugs has much-improved efficacy in as much as these drugs do not cross the blood brain barrier and thus do not cause adverse central nervous system effects such as drowsiness (Estelle *et al.*, 1999).

In this thesis, three classical H_1 antagonist drugs, promethazine, pheniramine and antazoline were utilised. Promethazine, a phenothiazine derivative is clinically used as a neuroleptic medication, anti-histamine and anti-emetic drug. This drug, however it has marked sedative effects. Promethazine is also used as an anaesthetic premedication to produce sedation, reduce anxiety (Lippmann *et al.*, 1978), or to reduce post-operative nausea and vomiting as dose-controlled transdermal device (Golembiewski *et al.*, 2005). This drug acts primarily as a strong H_1 receptor antagonist and a moderate muscarinic acetylcholine (mACh) receptor antagonist (Strenkoski-Nix *et al.*, 2000) and has a weak to moderate affinity for the 5-

hydroxytryptamine (5-HT) receptor (Fiorella *et al.*, 1995), and dopamine receptor (Seeman *et al.*, 1985), where it acts as an antagonist as well.

Antazoline is commonly used in eye drops, usually in combination with naphazoline, which is a vasoconstrictor and decongestant, to relieve symptoms associated with allergic conjunctivitis such as redness, puffiness, itchy and watering eyes associated with colds, allergies or eye irritations (Souri *et al.*, 2006).

Pheniramine, which also has anti-cholinergic properties is used to treat allergic conditions such as hay fever, urticaria and allergic conjunctivitis. However, sometimes pheniramine is also used as over-the counter sleeping pill (Wong *et al.*, 1981).

1.5.2 Cromoglycate-like anti-allergic drugs

The cromoglycate-like anti-allergic drugs include cromoglycate, nedocromil, pemirolast, lodoxamide, traxanol and amlexanox. These drugs are also known as ‘mast cell stabilizers’ as they are able to prevent mast cell degranulation (Figure 1.8). This family also includes some antagonists such as ketotifen, azelastine, olopatidine and epinastine, which appears to share a similar pharmacology with cromoglycate (Cook *et al.*, 2002).

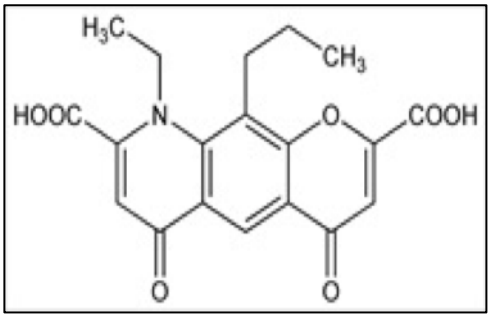
Cromoglycate sodium was first described by Altouynan in 1965 (Howell, 2005). It was derivative of a naturally-occurring cromone from the Egyptian plant Khella in which the active constituent ‘khellin’ was found to

have muscle relaxing properties (Kuzemko, 1989). Cromoglycate inhibits both the early and late phase of the asthmatic reactions (Rintala *et al.*, 2001) and clinical studies have indicated that this drug has significant clinical benefit in asthmatic children and adults and the side effects are minor (Auty, 1986; van der Wouden *et al.*, 2008). In animal models, cromoglycate has been shown to inhibit the allergic asthma and pulmonary inflammation (Abraham *et al.*, 1988; Abraham, 1989; Lapa e Silva *et al.*, 1995; Corbel *et al.*, 1999). Cromoglycate-like drugs have been shown to inhibit mediator action (Dahlén *et al.*, 1989), cytokine release (Rusznak *et al.*, 1996) and reduction in the infiltration of immune cells (Blondin *et al.*, 2003).

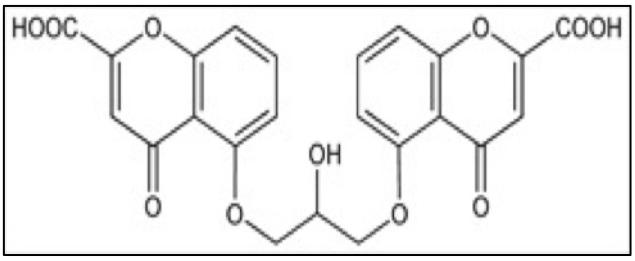
Nedocromil sodium was developed in the 1980s and was demonstrated to be an effective treatment for airway inflammation and to produce clinically relevant therapeutic effects even though these were rapidly reversible effects (Creticos, 1996). This drug exerts an inhibitory effect on bronchial hyper responsiveness as well as on activation of cells such as eosinophils and mast cells and thereby preventing the release of pro-inflammatory mediators. Nedocromil is also used as a clinical treatment for allergic conjunctivitis (Benbow *et al.*, 1993) as it significantly inhibits zymosan-induced complement activation when compared to other anti-allergic drugs such as azelastine or lodoxamide (Blondin *et al.*, 2003). Interestingly, some evidence also shows that these drugs not only target the mast cells but influence other facade of the inflammatory process both *in vivo* (Kuriyama *et al.*, 1986; Yazid *et al.*, 2010b) and *in vitro* (Yazid *et al.*, 2009).

Cromoglycate, nedocromil, lodoxamide, azelastine, olopatidine and ketotifen are used clinically in the UK for the treatment of mild to moderate asthma or for topical treatment of ocular and other allergic symptoms. Cromoglycate has also been used for treating ulcerative colitis (Grace *et al.*, 1987) and chronic enterocolitis (Rintala *et al.*, 2001). Even though the exact mechanism of action of mast cell stabilisers was not understood for many years, studies demonstrated that these drugs prevented the Ca^{2+} influx, characteristic of mast cell activation. Pemirolast was reported to inhibit IP_3 and Ca^{2+} mobilisation in antigen stimulated rat basophilic leukemia (RBL-2H3) cells (Fujimiya *et al.*, 1994).

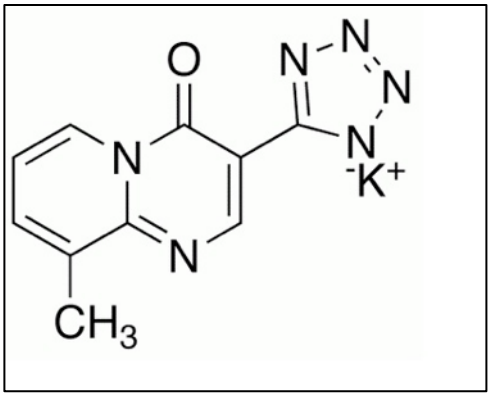
Despite its safety and efficacy, the cromones suffer from several drawbacks. The main limitation of these drugs is that their action is short-lived, thus the inhaled cromones need to be used four times daily, which is an inconvenient routine for chronic asthma patients. The inhaled cromones is less effective than the inhaled glucocorticocids although the former is more expensive than the latter (Barnes, 1997).



Nedocromil



Cromoglycate



Pemirolast

Figure 1.8: Structure of some of the cromoglycate-like drugs.

Among the family of the cromoglycate-like drugs, nedocromil and cromoglycate are the best know and most commonly used drugs pharmacologically for the treatment of ocular disease, asthma and food allergies.

1.5.2.2 Interaction between cromoglycate-like drugs and Anx-A1

Anx-A1 is found in substantial amounts in the granules of mast cells (Oliani *et al.*, 2000). Several studies have linked Anx-A1 and the mast cell function. Human recombinant Anx-A1 inhibited compound 48/80-induced paw edema (Cirino *et al.*, 1989) and the Anx-A1 peptide N-acetyl$_{2\text{-}26}$ suppressed mast cell degranulation, neutrophil and eosinophil migration in an allergic inflammation model in the rat pleural cavity and inhibited histamine release induced by ovalbumin in sensitised rats (Bandeira-Melo *et al.*, 2005). Upon LPS injury, mast cell degranulation was greatly enhanced in Anx-A1 null mice as compared to wild type (Damazo *et al.*, 2005).

Interestingly, it has been previously reported that cromoglycate-like drugs have been shown to bind with s100A11 proteins, which also bind to the annexin family, suggesting a potential interaction between these drugs and annexins. S100A11 is a homodimeric protein containing calcium-binding sites and it binds to the first 10-14 residues of Anx-A1 (Oyama *et al.*, 1997).

Work within our group has established that cromones inhibit PMN leukocyte activation in vivo (Yazid *et al.*, 2010a) and eicosanoid release by U937 cells (Yazid *et al.*, 2009). The cromone inhibitory effect depends on their ability to release Anx-A1 and is apparently caused by the inhibitory actions of the cromones on the phosphatase 2A (PP2A) enzyme, which normally terminates PKC activation.

However, these studies did not establish how endogenous Anx-A1 actually mediated the effects of cromoglycate-like drugs on mast cell mediator release.

1.5.3 Dual-action drugs

Even though anti-histamines have been effective in treating allergic inflammation, they are not able to inhibit all the manifestations caused by the allergic disease, since histamine is not the only mediator that is being released by the mast cells. Therefore, simultaneous development of mast cell stabilisers paved the way for the highly effective dual-action anti histamine/mast cell stabiliser drugs that are currently popular choices for treating allergic inflammation. These compounds were preferred as they offer more sustained and prolonged efficacy, due no doubt, to their ability to inhibit cytokine mediated inflammation including up-regulation of adhesion molecule expression and chemokine release (Cook *et al.*, 2002).

Dual-action drugs inhibit the degranulation of mast cells by preventing the cross-linking and activation of high affinity Ig-E receptor by allergen and thus interrupting the cascade of intracellular signalling. These drugs also have H_1 antagonist actions, thus exerting effects further down the allergic cascade of events by preventing the activation of histamine receptors on the proximal cells. These drugs have been demonstrated to have good efficacy and safety in the treatment of allergy and can therefore have an advantage as both prophylactic to prevent mast cell degranulation or as a therapeutic agent

to bring about symptomatic relieve (Chigbu, 2009)

Olopatidine, which is commonly used for the treatment of ocular allergy, has been shown to inhibit histamine, tryptase, PGD_2 and TNFα in human conjunctival mast cells (Leonardi *et al.*, 2010). Another study has shown that ketotifen is more potent than olopatidine in inhibiting histamine release from mast cells, however ketotifen prevents mediator release at a concentration greater than the maximal effective dose, thus affording a smaller safety margin than olopatidine (Yanni *et al.*, 1996). Ketotifen also inhibited chemotaxis and activation of eosinophils in vitro (Podleski *et al.*, 1984). Epinastine, which is another 'dual-action' drug used in this study, also inhibits the release of inflammatory mediators from mast cells, eosinophils, neutrophils (Amon *et al.*, 2000) as well as oxygen radical generation by inflammatory cells (Fukuishi *et al.*, 1995). Allergic rhinitis has been treated clinically with orally administered epinastine with minimal adverse effects (Abelson *et al.*, 2004).

It is noteworthy that mast cell heterogeneity exists between different tissues within the same species or other species, which causes the cells to respond differently to various secretagogues and drugs. Therefore drugs, which are effective on mast cells from one species, might not exert the same effect on mast cells retrieved from other source.

1.6 SCOPE OF THESIS

1.6.1 Hypothesis

Based on previous research highlighting the role of Anx-A1 in several models of allergic inflammation, the following hypothesis was formulated: *anti-allergic drugs exert their effects on mast cells by stimulating the release of the anti-inflammatory endogenous protein Anx-A1 which acts through the FPR to prevent mediator release.*

1.6.2 Aims

Although the anti-allergic drugs have been used for the treatment of allergy and asthma for many years, their mechanisms of pharmacological action have not been convincingly explained. Thus, this hypothesis will be challenged by addressing the following aims.

Therefore, the first aim of this thesis was to investigate the ability of various anti-allergic drugs to induce Anx-A1 phosphorylation in U937 cells. As this rapid phosphorylation event is crucial for the translocation and export of Anx-A1 into the extracellular milieu, it is important to categorise the drugs on this ability. U937 cells were chosen because these cells are easy to culture, contain abundant levels of Anx-A1 and they respond well to anti-allergic drugs treatment.

Since the main target for anti-allergic drugs are the mast cells, cord-blood derived mast cells (CDMCs) and bone marrow derived murine mast

cells (BMDMCs), stimulated by compound 48/80 were utilised to investigate the next aims. It has been established that GCs activate PKC in U937 cells (Yazid *et al.*, 2009), thus using biochemical and immuno-fluorescent techniques, the possible interaction between cromoglycate-like drug, nedocromil and the dexamethasone to activate PKC, thereby potentiating Anx-A1 phosphorylation and release was investigated. To further confirm if the other anti-allergic drugs, such as ketotifen and promethazine has similar mechanism of action as nedocromil, their ability to promote Anx-A1 phosphorylation was demonstrated.

To address the notion that the ability of the anti-allergic drugs to release Anx-A1 is critical for their acute mediator inhibitory effects in mast cells, the neutralising anti-Anx-A1 monoclonal antibody and the BMDMCs isolated from Anx-A1 null mice was utilised. The inhibitory effects of anti-allergic drugs on the release of mast cell mediators such as β-hexosaminidase, tryptase, histamine and PGD_2 in activated CDMCs were analysed.

The next aim was to explore the possibility that FPR2, which is one of the receptor for Anx-A1, might be involved in the mechanisms of action of cromones. To address this aim, the FPR2 antagonist, WRW4 peptide in CDMCs and the BMDMCs isolated from fpr2/3 null mice were utilised to ascertain the role of FPR2 in the acute effects of nedocromil.

Since mast cell degranulation is governed by the signaling pathway, the MAPK downstream signaling that contributes to the ‘mast cell stabilising’

effects of nedocromil was determined and also the role of Anx-A1 to elicit this pathway was investigated. Western blots were performed to analyse the phospho-p38 and phospho-JNK in stimulated CDMCs pre-treated with nedocromil.

2. MATERIALS AND METHODOLOGY

2.1 MATERIALS

Nedocromil sodium was generously supplied by Sanofi-Aventis (Paris, France). Isotype matched irrelevant (IgG1) was obtained from ABD Serotec, Oxford, UK. Human recombinant protein Anx-A1 was a gift from Dr F. D'Acquisto. An in-house specific neutralising anti-Anx-A1 (clone 1B; 20 µg/mL) was used. Unless otherwise stated, all materials were purchased from Sigma-Aldrich (Poole, UK).

2.2 METHODOLOGY

2.2.1 Cell culture

2.2.1.1 U937 human pro-monocytic cell line

U937 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 (Invitrogen, Paisley, UK), which were supplemented with 10% Fetal Bovine Serum (FBS; Gibco®, UK), 1% L-glutamine, 1% non essential amino acids and 0.1% gentamycin, at 37°C with 5% CO_2 atmosphere. The culture flasks (Nunc, Thermo Scientific, Hempstead, UK) containing proliferating pro-monocytic cells were sub-cultured when the cells were grown to 70-80% confluency. Analysis of proteins by Western blot, proliferating cells were transferred from flask into a 12-well plate at a density of 1 million cells per well. Upon plating, these cells were incubated in the presence of 10ng/ml phorbol 12-myristate 13-acetate

(PMA) for 24 h to acquire monocytic-like properties after which they become responsive to glucocorticoids (Solito *et al.*, 1991a). U937 cells were chosen instead of other cells because U937 cells have been demonstrated to be responsive to both steroids and cromoglycate-like drugs (Yazid *et al.*, 2009). The addition of PMA to U937 cells for 24 h resulted in a 2.4 fold increase in the cell surface level of Anx-A1 and inhibition of the ATP-induced Ca^{2+} response, thus making these cells responsive to steroids (Willmott *et al.*, 1997). Although PMA stimulates the activation of PKC, the incubation of U937 cells with PMA for 24 h has been shown not to influence the PKC dependency effects in these cells (Solito *et al.*, 1991b).

2.2.1.1.1 Passaging U937 cells

10ml of the cell suspension in medium was centrifuged at 1100 g for 5 min. Cell pellet was resuspended with 20ml of new medium and transferred into a T75 flask.

2.2.1.1.2 Preparation of drugs

Dexamethasone, promethazine, pheniramine, antazoline, nedocromil, cromoglycate, pemirolast, ketotifen, olopatidine and epinastine were prepared at 10μM (stock solution; dissolved in medium) and serial dilutions were prepared so that the final concentrations of the drugs were in the range of 2-500nM. Water-soluble salts of the drugs were chosen for convenience. In some cases, the drug-treated cell aliquots were incubated for 20 min with a well-characterised (D'Acquisto *et al.*, 2008) specific neutralising anti-Anx-A1

monoclonal antibody (clone 1B; 20 µg/mL), or isotype matched irrelevant (IgG1, ABD Serotec, Oxford, UK), monoclonal antibodies. Specific neutralising anti-Anx-A1 monoclonal antibody (was previously manufactured by Biogen Corp, Cambridge, MS, USA, but upon acquiring the clone (1B), this antibody has been produced in-house. This antibody is targeted to detect the N-terminus of Anx-A1 and no cross-reactivity has been reported with this antibody. Untreated mentioned in all the experiments means that the cells were incubated with media alone.

2.2.1.2 Cord blood-derived human mast cells (CDMCs)

$CD34^+$ stem cells (Lonza, UK) were cultured in Stemspan serum free media containing 100ng/ml SCF and 50ng/ml IL-6, with 1 ng/ml IL-3 (Peprotech, London, UK) added during the first 2 weeks. Medium supplemented with IL-6 and SCF was added weekly to the cells, with 10% FBS added from week 9. Cells were used for experiments between 11-18 weeks of culture (Dahl *et al.*, 2002). At week 8, un-stimulated CDMCs were immunophenotyped as >90% c-kit positive by FACS analysis. To further confirm the morphology, these cells were stained with toluidine blue which stains the metachromatic granules of the mast cells. The cells were maintained at a density of $1.0x10^6$ cells/ml for no longer than 15 weeks.

2.2.1.3 Bone marrow-derived murine mast cells (BMDMCs)

To generate primary bone marrow-derived murine mast cells (BMDMCs), femur bones from Anx-A1$^{-/-}$ BALB/C, FPR$^{-/-}$ C57/B6 and their respective WT mice (4-6 weeks old, Charles River, Kent, UK) were used. Animal work was performed according to UK Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986) and was approved by the Queen Mary University of London Ethics Committee (London, UK).

2.2.1.3.1 Preparation of BMDMCs

The donor mice were sacrificed by cervical dislocation. The mice and the surgical instruments were rinsed with 70% ethanol as needed prior to bone marrow extraction. The skin from the legs was removed using sterile forceps and scissors. The femur and tibia were removed and the tissue was scrapped away from bones using sterile scissors and scalpel blades.

The bones were kept in PBS on ice until all the bones were collected. By using sterile instruments, the bone is picked up with forceps and the epiphysis of the bone was cut with scissors to expose medullary cavity. The marrow was flushed out of each bone with flushing medium (DMEM, 10% FBS, 2mM L-glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin, 0.1 mM non-essential amino acids and 50µM β-mercaptoethanol) using a 5ml syringe with a 25-gauge needle into a labeled 50ml Falcon collection tubes. The femoral and tibia bone marrow cells from each mouse were pooled into one collection tube.

The bone marrow cells were gently aspirated and dispersed to generate a single cell suspension and were passed through a cell strainer (70µM). The cells were then centrifuged (1000g for 5 min, 20°C) and the supernatant were removed and the pellet was resuspended in 10ml of culture medium which consist of DMEM (Invitrogen, Paisley, UK), 10% FBS, 2mM L-glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin, 0.1 mM non-essential amino acids and 50µM β-mercaptoethanol, 5 ng/ml of r-murine IL-3

and 10ng/ml SCF (PeproTech, London, UK). The cells were placed in tissue culture flask of appropriate size (e.g., T25 for 1 mouse or T75 for 2 mice).

2.2.1.3.2 Cell culture of BMDMCs

The medium and suspended cells were transferred into a new flask after 1-2 days to remove debris and adherent cells. Fresh culture medium was added into the flask. In subsequent weeks, the culture medium was added every 3-4 days to maintain a cell density of $2.5x10^5$ – $1x10^6$ cells/ml. The suspended cells were transferred into a new flask once a week until no further adherent cells was observed in the culture flask. The differentiation of BMDMCs takes 4-6 weeks and phenotypic maturity of these cells was tested before using the cells for *in-vitro* experiments.

2.2.1.4 Assessing phenotypic maturity of CDMCs and BMDMCs

2.2.1.4.1 FACS Staining for c-kit (CD117)

CDMCs or BMDMCs were diluted in PBC (PBS + 0.15% BSA and 1.0mM $CaCl_2$) buffer and plated in duplicate in a density of $1x10^5$ cells per well in a 96-round bottom plate. Cells were then washed three times by centrifugation with PBC at 1000g for 30 sec. Negative control wells contained 20µl of IgG and 20µl of PBC whereas the isotype control wells had 20µl of isotype human IgG and 20µl of PBC. 20µl of IgG and 20µl of c-kit (CD117) antibody (Chemicon International, USA) were added to the sample wells. The plate was incubated on ice for 45 min. The washing procedure was repeated

twice with PBC and once with PBS. 200µl of PBC solution was added to the wells to resuspend the cells, which were then analysed using the FACS Caliber (Becton Dickinson).

2.2.1.4.2 Assessing the degranulation of CDMCs and BMDMCs upon compound 48/80 stimulation using toluidine blue staining

Toluidine blue staining was used to distinguish the metachromatic granules of the mast cells. Toluidine blue is a small weakly hydrophilic cationic dye, which turns blue when attached to DNA or RNA in chromatin, or purple when it is in contact with glycosaminoglycans in mast cell granules (Sridharan *et al.*, 2012). CDMCs or BMDMCs were plated in duplicate at $1x10^5$ cells per well in a 96-well plate. Cells were washed twice in cold 2% FBS-PBS and resuspended in 100µl of cold 1% BSA in PBS. All the samples were kept on ice. Since the CDMCs and BMDMCs were in suspension the cells were deposited onto a slide using the cytospin at 2500g for 3 min. Slides were the left to air-dry prior to the removal of filters from the slides. Slides were then examined under microscope for proper adherence of cells. The slides were flooded with 0.5% of toluidine blue solution for 5 min and rinsed three times with ddH_2O. Cover slips with mounting medium were placed over the specimen and viewed under the light microscope at 60x magnification (Figure 2.1).

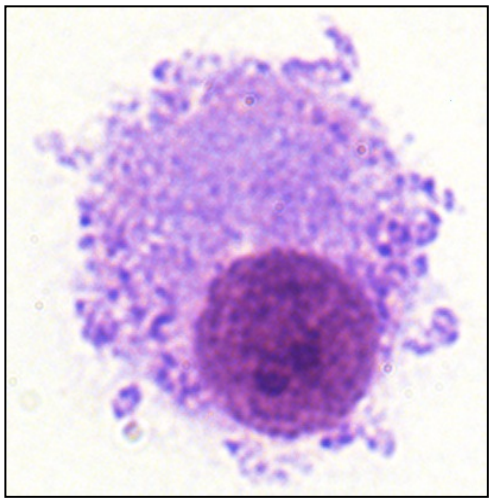


Figure 2.1: Unchallenged cord-blood derived mast cell (CDMC) stained with toluidine blue.

The metachromatic granules in the cell are stained red-purple confirming the mast cell phenoype.

2.2.1.5 Treatment with drugs

CDMCs/BMDMCs were stimulated with Compound 48/80 (10µg/ml) for 10 min at 37°C with 5% CO_2 atmosphere. Drugs or antibodies, to be tested in this system, were added 5 min prior to the addition of Compound 48/80. Samples were centrifuged at 1000g for 5 min before the cell-free supernatants were collected to measure histamine and PGD_2 release. Cell lysates and supernatant were prepared for Western blots. Sample aliquots were stored at -80°C for further analysis.

2.2.1.6 Cryopreservation of cells

Confluent cells were counted, sedimented by centrifugation, aliquoted at 10^6/vial, and cryopreserved in 1ml of solution containing 90% FBS and 10% dimethyl sulphoxide (DMSO). The cells were frozen slowly at -80°C overnight before being transferred into a liquid nitrogen container.

2.2.1.7 Thawing of cryopreserved cells

Vials of frozen cells were removed from the liquid nitrogen tank and thawed immediately with 10ml of complete medium. The cells were washed by centrifugation at 1000g for 5 min to ensure complete removal of DMSO. Cell pellets were then resuspended with 10ml of complete medium and seeded into T25 flasks.

2.2.1.8 Counting of cells

Cells were counted using a Nueberg haemocytometer (Figure 2.2). The haemocytometer was cleaned with 70% ethanol prior to fixing the cover slip. 10µl of cells were diluted with 990µl of Trypan Blue (1 in 100 dilution). 10µl of sample were transferred into the haemocytometer by pipette, and the cells were counted from the 4 quadrants of haemocytometer using a counter. 90% of the cells were viable upon determination by trypan blue. Total number of cells was determined using the formula below:

Average of cells in 4 quadrants x dilution factor (100) x 10^4 = Number of cells/ml

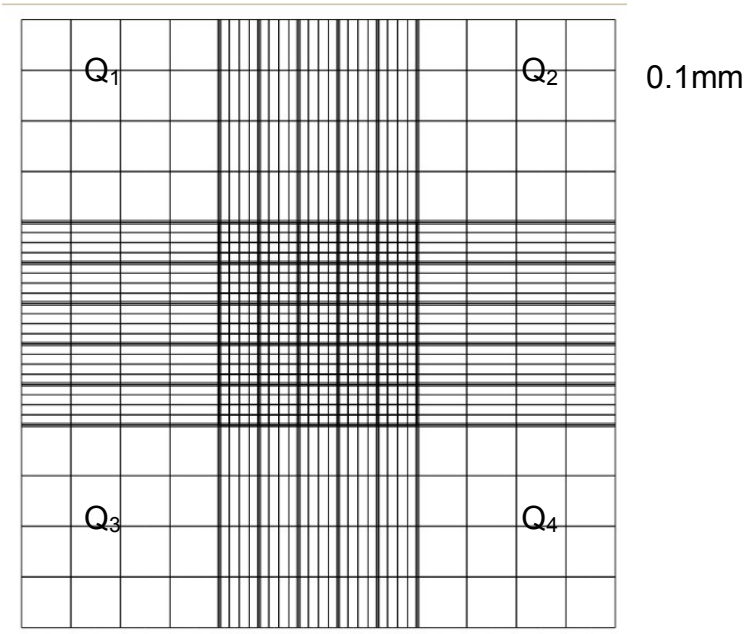


Figure 2.2: Illustrations of the counting grid on a Neubauer haemocytometer.

Cells were counted in the four quadrants and an average was obtained.

2.3 CHALLENGE OF CDMCS AND BMDMCS

2.3.1 DNP-Ig-E activation

BMDMCs were incubated overnight with anti-mouse monoclonal dinitrophenyl (DNP) - IgE (100 ng/mL; Sigma) to sensitise the cells and the following day they were activated by adding DNP-BSA (1 µg/mL; Sigma-Aldrich, Dorset, UK). Cell-free supernatants were collected at 1h to measure histamine and/or PGD_2 release. Aliquots were stored at -80°C for subsequent analysis. When drugs or antibodies were tested, these were added to cells 5 min prior to DNP/IgE cross-linking.

2.3.2 Compound 48/80 stimulation

Aliquots of CDMCs and BMDMCs were stimulated with compound 48/80 (10 µg/ml; Sigma-Aldrich, Dorset, UK) for 10 min at 37°C. Drugs or antibodies were added to the cells 5 min prior to challenge. Cell-free supernatants were taken to measure histamine and/or PGD_2 release. Aliquots were stored at -80°C for subsequent analysis.

2.4 ANALYTICAL METHODOLOGY

2.4.1 Chromatographic purification of antibody.

Phosphorylated forms of Anx-A1 were detected using a purified specific antibody raised in rabbit against Anx-A1 phosphorylated at Ser^{27} (Solito *et al.*, 2003c). MAbTrap kit Protein G affinity column (GE Healthcare, Buckinghamshire, UK) was used for the purification of monoclonal and polyclonal IgG from the rabbit serum. The rabbit serum was a kind gift from Dr Egle Solito.

The column and buffers were warmed to room temperature prior to the purification process. Binding (2.5ml binding buffer concentrate + 22.5ml of dH_2O to a total volume of 25ml) and elution buffer (0.5ml binding buffer concentrate + 4.5ml high quality water to a total volume of 5ml) were diluted. Collection tubes were prepared by adding 60-200µl of neutralising buffer per ml fraction to be collected. This allow for immediate renaturing of the purified IgG to preserve the activity of labile IgGs.

The samples were centrifuged (1000g for 5 min) and filtered (0.45µm filter) to increase clarification and prevent back- pressure problems. The filtered serum then was diluted 1:1 with prepared binding buffer. The syringe or pump tubing were filled with distilled water and the top-cap was removed and the column and the syringe were connected through the luer connector. The connection is made to 'drop by drop' to avoid introducing air into the column. The snap-off end at the column outlet was removed. Ethanol

preservative were washed out with 5ml of distilled water at ~ 1 drop/sec. The column was equilibrated with at least 3ml of binding buffer. The sample was applied using the syringe or by pumping onto the column. Thereafter, the column was washed with 5-10ml binding buffer or until no material appears in the effluent.

Finally the elution buffer (3-5ml) was used to elute the sample. The protein concentration of the purified antibody was determined by using the nano drop (ND-100, LabTech) and resulted in a corresponding yield of 0.88mg of pure rabbit polyclonal IgG1. The working dilution (1:1000) of this antibody (Anx-A1-P-Ser^{27}) was optimised using the Western blots. The specificity of the antisera raised against phosphorylated Anx-A1 proteins was confirmed by the findings in Western blot and this antibody did not cross-react with the unphosphorylated protein human recombinant Anx-A1$_{1–188}$ (5 µg/lane).

2.4.2 WESTERN BLOTTING PROTOCOLS

2.4.2.1 Protein extraction

Upon treatment with stipulated drugs, cells were centrifuged at 1000g for 5 min. Cell-free supernatant were removed, labelled and stored at -80°C for further analysis. Lysis buffer (100µl) was added to each sample for 10 min. Lysis buffer was prepared in Tris-HCl (20mM) and is composed of NaCl (200mM), Tris X-100 (1%), 1mM EDTA to remove Anx-A1 attached to the cell surface, and a protease inhibitor cocktail comprising of aprotinin (0.03%), Na_3VO_4 (1mM), leupeptin (10uM), sodium fluoride (1mM) and PMSF (200µM).

Protein extraction was performed on ice to retard any enzymatic digestion of extracted protein. Following the 10 min incubation with the lysis buffer, cells were scraped with cell-scraper and passed vigorously through a 25G needle attached to a 1ml syringe and collected into an eppendorf tube. The samples were centrifuged at 11000xg for 10 min and the supernatant was collected into new tubes. Prior to performing Western blots, protein assay was carried out to determine the amount of protein in the samples.

2.4.2.2 Protein assay

Total protein concentration of samples was determined by a colorimetric protein assay (BioRad, USA). This assay was based on the dye-binding assay in which a differential colour change of a dye occurs in response to various concentrations of protein. The reduced species has a characteristic blue colour with maximal absorbance at 595nm and minimum absorbance at 465nm. Samples were prepared in x10 dilution for this assay.

A linear range of 0.05 - 0.5 mg/ml of BSA standard was prepared. In accordance to the manufacturer's protocol, 10µl of standards and samples were added in triplicates in 96-well plate. The dye reagent was prepared by adding 1 part of reagent concentrate with 4 parts of ddH_2O. 200µl of the diluted dye was added to the standards and samples and read at 595 nm using a microplate reader (Titertek^{TM}, Vienna, Austria). Protein concentrations in each sample were extrapolated from the standard curve using the GraphPad Prism Software version 4 for Mac (GraphPad Software, San Diego, California, USA, www.graphpad.com).

2.4.2.3 SDS-polyacrylamide gel electrophoresis

Total proteins were separated by discontinuous SDS-PAGE according to the method of Laemmli (1970), under reducing conditions. As SDS is an anionic detergent, it imparts a negative charge to all proteins. Therefore, protein separation is based on molecular weight. The discontinuous stacking

gel is composed of a 4% acrylamide gel, where proteins are concentrated, and a 10% or 12% acrylamide resolving gel where proteins are separated. Thus, the resolving gel acrylamide is selected based on the range of sizes of protein that needs to be analysed. The composition of the resolving and stacking gels are detailed in Table 2.1.

The gels were poured into a gel sandwich made of 1 white gel holder and 1 glass holder separated by 1.5mm spacers. Resolving gel were made first and left to polymerize for 20 min before adding the stacking gel. A thin layer of water was added onto the resolving gel to release any air bubbles that might have been trapped during the polymerization process. Before the addition of stacking gel, the layer of water is gently tapped off the gel sandwich. The stacking gel was then poured into the casting frame and left to polymerise for 30 min. Thereafter, a 10-well comb was carefully inserted to avoid forming bubles. Once the gel has hardened, either it could be used immediately or it is transferred into a wet tissue and kept in the fridge at 4°C until required.

Composition	Resolving Gel		Stacking gel (4%)
	10%	12%	
30% (w/v) Acrylamide: 0.8% Bis acrylamide solution (Protogel)	13.3 ml	16 ml	3.9 ml
Resolving gel buffer (1.5M Tris-HCl, pH 8.8 0.4%SDS	10.4 ml	10.4 ml	-
Stacking gel buffer (0.5M Tris-HCl, pH 6.8 0.4%SDS	-	-	7.5 ml
10% (w/v) Ammonium Persulphate (APS)	400 µl	400 µl	150 µl
dH_2O	15.8 ml	13.2 ml	18.3 ml
Tetramethylethylenediamine (TEMED)	40 µl	40 µl	30 µl

Table 2.1: Composition of resolving and stacking SDS-PAGE gels.

The volume stated is sufficient to make 4 gels.

2.4.2.4 Western blotting

30µg of proteins and 6x Laemmli sample buffer (36% (w/v) glycerol; 60% (w/v) 4x Tris-HCl/SDS, pH 6.8; 4% (w/v) distilled H_2O; 0.1 g/ml SDS; 0.093 g/ml dithiothreitol; 0.12 mg/ml bromophenol blue) were mixed and denatured by warming at 80°C for 5 min. The prepared gels were assembled into a chamber and submerged in electrophoresis running buffer (25mM Tris Base, 200mM glycin, 0.1% SDS, Sigma). Kaleidoscope pre-stained standards (GE Healthcare, Buckinghamshire, UK) were used to determine the molecular weight.

Electrophoresis was carried out at 80 Volts for the first 20 min and then increased to 120 Volts once the sample had passed the stacking gel. Electrophoresis was stopped once the samples had reached the bottom of the gel. A PVDF Immobilon membrane was activated in methanol prior to the transfer. The proteins were then electrophoretically transferred onto the membrane. The gel sandwich was prepared in a cassette by layering sponge pad, filter paper, gel, PVDF membrane, filter paper and sponge pad respectively in this manner (Figure 2.3).

The sandwich cassette was then placed in a tank set in a cold room submerged in chilled transfer buffer (25mM Tris base. 192mM glycine, 20% methanol). The transfer was achieved by application of 100 Volts for 90 min. After the transfer, gels were stained with Coomasie blue to ensure that no protein is left on the gel and therefore equal amount of protein had been

transferred onto the membrane.

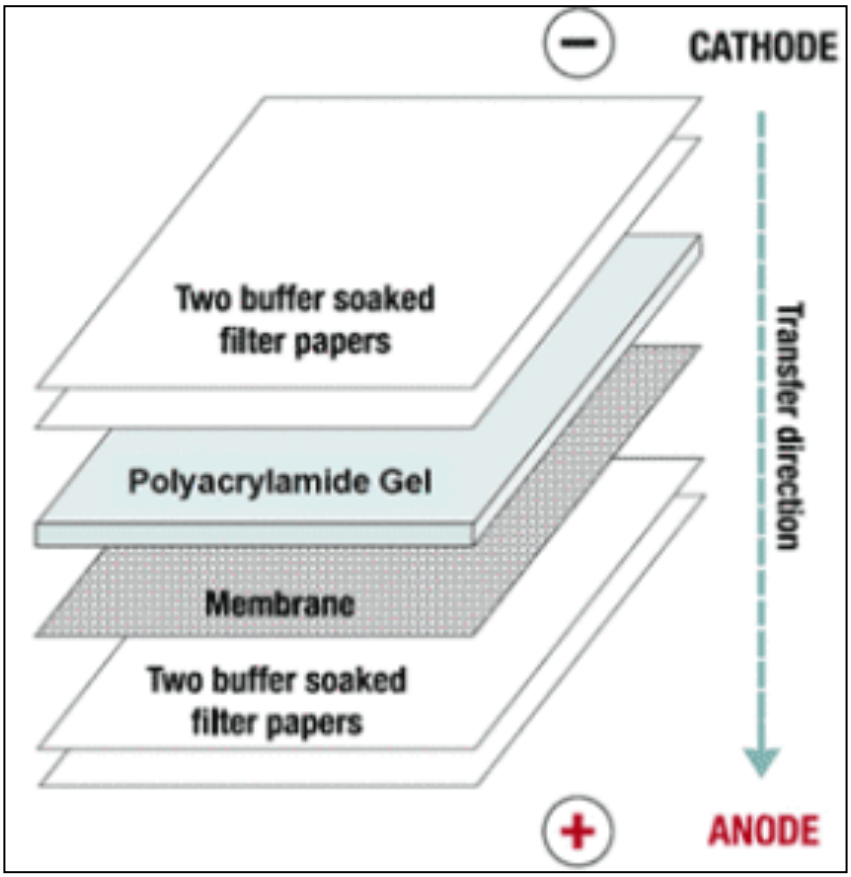


Figure 2.3: Arrangement of the transfer stack.

The protein from the polyacrylamide gel is transferred onto the PVDF membrane using a semi-dry transfer system, which consists of 'sandwich' stack soaked in transfer buffer.

2.4.2.5 Immunodetection of proteins

Non-specific binding sites were blocked by incubation of the blots in 5% non-fat dry milk or BSA in Tween 20-Tris Buffered Saline (TTBS; 150mM sodium chloride, 2mM Tris base, pH 7.4 containing 0.1% Tween 20, Sigma) according to the antibody manufacturer's protocol on a rocking platform for 1 h at room temperature or overnight at 4°C. Membranes were then incubated with specific primary antibody (Table 2.2) for 1 - 2 h at room temperature or overnight at 4°C. The membranes were washed three times for 10 min each with TTBS to ensure removal of unbound antibody. After washing, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary species-specific antibody (Table 2.3) for 1 h at room temperature.

Antigen	Host	Dilution	Type	Source	Cat No	Concen-tration
Anx-A1 (1B)	mouse	1:2000	Monoclonal	In-house	Clone 1B	10µg/ml
Anx-A1	rabbit	1:2000	Polyclonal	Invitrogen	71-3400	5µg/ml
Ser^{27}-Anx-A1-P	rabbit	1:1000	Polyclonal	Neosystems, Strasbourg, France	nil	10µg/ml
Pan P-PKC, P-$PKC_{\alpha\beta}$, P-PKC_{δ}, P-PKC_{σ}, total PKC	rabbit	1:1000	Polyclonal	Cell Signalling Technology	9371 9375 9374 9376 2056	10µg/ml
Tryptase	mouse	1:1000	Monoclonal	Abcam	Ab-2378	10µg/ml
α-tubulin	mouse	1:5000	Monoclonal	Sigma Aldrich Technology	T5168	2µg/ml

Table 2.2: Primary antibodies used for Western blot.

Target IgG	Host	Dilution	Type	Source	Cat No	Concent-ration
Mouse	goat	1:5000	Polyclonal	Dako Cytomation	P04470 1-2	2µg/ml
Rabbit	goat	1:2000	Polyclonal	Dako Cytomation	P04480 1-2	5µg/ml

Table 2.3: Secondary antibodies used for Western blots.

2.4.2.6 Enhanced chemiluminescence detection

Immunoreactive bands were visualised using an enhanced chemiluminescence (ECL) kit. ECL solution 1, which contains 1ml Luminol stock (250mM in DMSO, Sigma), 0.44ml p-coumaric acid stock (0.29/20ml dH_2O, Sigma), 10ml 1M Tris base (pH 8.5) and 88.56 ml dH_2O was prepared. ECL solution 2 contained 64µl of 30% H_2O_2, 10ml 1M Tris Base (pH 8.5) and 90ml dH_2O. Equal volume from ECL solution 1 and 2 were mixed and the immunoblots were submerged in this solution for 1 min to activate a chemiluminescence reaction. The excess fluid was drained and immunoblots were wrapped in plastic film prior to exposure to X-ray Hyperfilm ECL (Amersham, Pharmacia Biotech). The films were developed using the Jet X-Ray SRX 101A Developer (Jet X-ray, UK). Immunoblots were kept wet and placed at 4°C until being stripped or reprobed. The illustrations of ECL reaction is depicted in Figure 2.4

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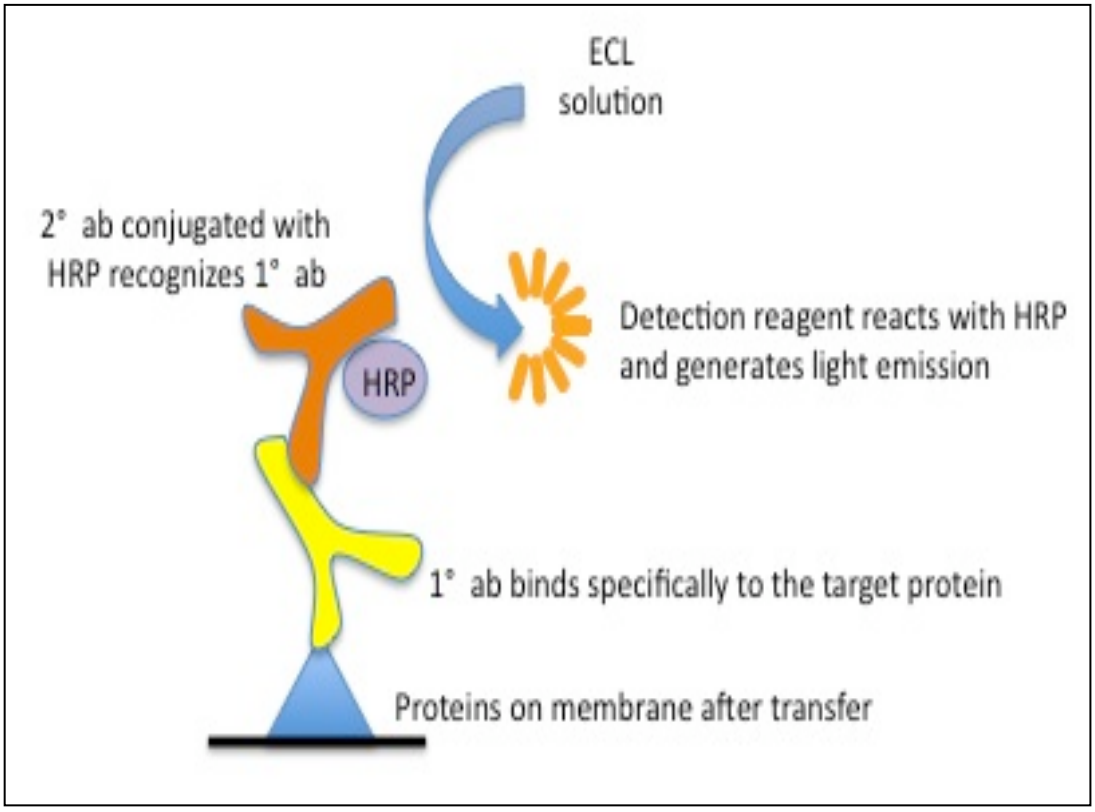


Figure 2.4: Schematic illustrations of ECL reaction.

Chemiluminescence occurs when a chemical reagent containing stored energy releases light. The reagent is normally stable and does not emit light, but can be converted into a light-emitting product, for example after interaction with a specific enzyme. In most contemporary ECL systems, the enzyme horseradish peroxidase (HRP) conjugated to a secondary antibody is utilised to convert the substrate, producing light, which is then captured onto an autoradiographic film. The intensity of the light is proportional to the quantity of HRP enzyme and hence the protein of interest.

2.4.2.7 Stripping the membrane

To recycle and probe the membrane with different antibodies, the membrane was stripped using a mild stripping solution (1.5g glycine, 0.1g lauryl sulphate and 1ml Tween 20 made up in 100ml dH_20). Briefly, the membrane was washed once with TTBS and submerged in stripping buffer for 30 min at room temperature with gentle rocking. The membrane was then washed three times for 5 min in TTBS before being exposed to ECL detection in order to ensure complete removal of previous primary and secondary antibodies. Membranes were then washed with TTBS, blocked and re-blotted with primary antibody. Membranes were not stripped more than three times.

2.4.2.8 Band quantification

Western blotting films were scanned at high resolution and the images were downloaded using Image J software and the density of each band assessed and the protein band was normalised against the α-tubulin band of the same sample. Each Western blot was repeated three times and the densitometry results averaged.

2.5 Immunoassays

2.5.1 Principal of the ELISA

Enzyme linked immunosorbent assay (ELISA) is an immunoassay used to detect specific proteins. In a sandwich ELISA, the antigen of interest is bounded by two antibodies: the primary capture antibody, which is bound to the plate and the secondary, detection antibody found in the solution (Figure 2.5). The antigen of interest must therefore contain at least two distinct antigenic sites or epitopes. The sequence of ELISA is as follows:

1. Binding of capture antibody to 96-well plate.
2. Blocking of non-specific sites.
3. Binding of antigen to capture antibody.
4. Binding of secondary antibody to antigen.
5. Horseradish peroxidase (HRP) conjugated secondary antibody catalyses an enzymatic colorimetric reaction.
6. Reaction is stopped at an appropriate time.
7. Colour intensity is directly proportional to antigen concentration and is read on a spectrophotometer (maximum absorbance at 450nm). Cell supernatants for assays were collected and stored at -80°C prior to analysis.

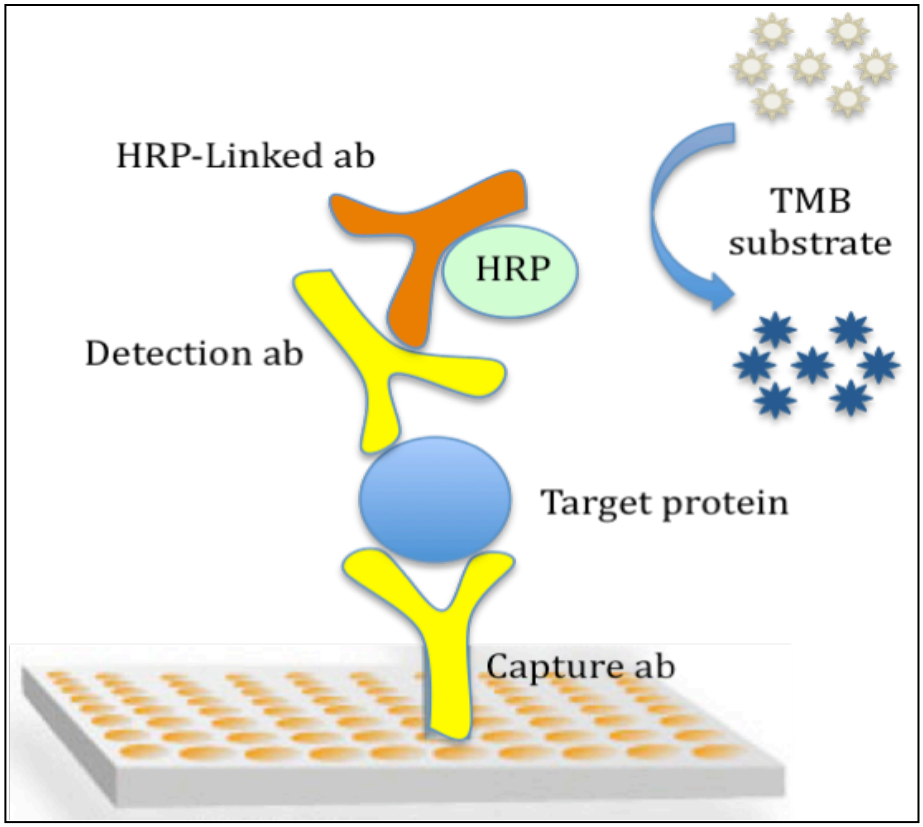


Figure 2.5: Illustration of a 'sandwich' ELISA.

The assay involves the capture of a multivalent antigen or target protein in a sandwich complex by a capture antibody adsorbed onto a coated plate and a detection secondary antibody. The secondary antibody is further detected by an HRP-conjugated tertiary antibody. HRP will convert a transparent TMB substrate into a coloured product which absorbance will be directly proportional to the amount of antigen present in the sample.

2.5.2 Measurement of Thromboxane B_2 (TxB_2)

Commercially available enzyme immunoassay was used to detect and quantify TxB_2 released in the supernatant (BioTrak Assay, Amersham, UK). The method was performed following the manufacturer's instructions. A standard curve ranging from 0.5 - 6.4 pg/well was prepared using the reagent provided, 50µl of standard was added to the plate in duplicate and 50µl of the unknown samples were added into the appropriate wells, for 1 h incubation.

The microtiter plate was pre-coated with donkey anti-rabbit IgG so that the rabbit anti-TxB_2 binds to the immobilized antibody. This incubation allowed the unlabelled antigen present in the cell-free supernatants to bind to the resultant complex and to compete for a limited number of binding sites with the labelled peroxidase-TxB_2 added in each well. Any sample that was unbound was removed by washing.

The amount of peroxidase-TxB_2 labelled bound to the antibody was determined by the addition of 150µl of a tetramethylbenzidine (TMB)/hydrogen peroxide enzyme substrate. After 15 min shaking at room temperature, the reaction was halted by the addition of 100µl of 1M sulfuric acid, which turns the sample from blue to yellow colour. The optical density was then read at 450nm in a microplate reader within 30 min.

2.5.3 Histamine assay

CDMCs and BMDMCs were pre-treated with anti-allergic drugs or antibodies for 5 min and stimulated with compound 48/80 (10μg/ml) for 10 min before the cell-free supernatants, and in some cases, the total cell lysate were collected for histamine determination. Histamine release was measured by a commercially available ELISA kit (SPI Bio, Bertin Pharma). The assay was performed according to the manufacturer's protocol.

This assay is based on the competition between unlabelled derivatised histamine and acetylcholinesterase (AChE)-linked histamine (tracer) for limited specific mouse anti-histamine antibody sites. Histamine is derivatised to increase the affinity for the histamine to the antibody and consequently increase the sensitivity of the assay. Standard concentrations were prepared respectively (0.39 – 50 nM) using the same culture medium as the samples. Derivatization reagent was reconstituted with 1mL of N-N-dimethylformamide (DMF). 200μl of standards and samples were derivatised with 50μl of derivatisation buffer and 20μl of the derivatization reagent and vortexed immediately. Whereas, 200μl of assay medium was used for the estimates of maximum binding evaluation.

After the derivatisation process, the microtiter plate was washed five times with wash buffer. 100μl of the prepared derivatised solution of standards, samples and maximum binding was dispensed into the wells according to the plate set-up. The plate was covered and incubated for 24 h

at 4°C. Prior to the developing process, the plate was washed five times with 300µl of wash buffer. 200µl of Ellmans reagent was added into the wells and incubated with an orbital shaker in the dark at room temperature. The plate was read between 405 and 414nm using the spectrophotometer. The net histamine release (%) was calculated following the equation:

$$\textbf{Net histamine release (\%)} = \frac{\text{Challenged release (nM)} - \text{spontaneously release (nM)}}{\text{Total histamine content (nM)} - \text{spontaneously release (nM)}} \times 100\%$$

The spontaneous histamine release (%) was calculated following the equation below:

$$\textbf{Spontaneous release (\%)} = \frac{\text{Histamine release (nM)}}{\text{Total histamine content (nM)}} \times 100\%$$

2.5.4 PGD_2 assay

Cell-free supernatants from CDMCs and BMDMCs were collected and processed for the PGD_2 assay immediately. The PGD_2 assay (Cayman Chemical, US) was performed according to the manufacturer's protocol. This assay is based on the competition between PGD_2–acetylcholinesterase (AChE) conjugate (PGD_2 tracer) for a limited number of PGD_2 monoclonal antibody binding sites. As the concentration of PGD_2 tracer is held constant while the concentration of PGD_2 varies, the amount of PGD_2 tracer that is able to bind to the monoclonal antibody will be inversely proportional to the concentration of PGD_2 in the well. Buffers and reagents used in this assay were prepared according to the protocol.

The standards were in the range of 117.2 - 15000 pg/ml and were diluted with culture medium. The plate was pre-coated with goat anti-mouse polyclonal antibody and blocked with proprietary formulations of proteins. This plate contains blanks, non specific binding (NSB) wells, maximum binding wells, standards and samples which were assayed in duplicates. For NSB wells, 50µl of EIA buffer and 50µl of culture medium were added and the maximum binding (B_0) wells contain 50µl of culture medium only. 50µl of standards and samples were added in duplicate into the appropriate wells. 50µl of PGD_2 Tracer were added into all wells except the blanks. Subsequently, 50µl of prostaglandin D_2 monoclonal antibody was added into all wells except NSB and blank wells. The plate was then covered with plastic

film and left to incubate for 2 h at room temperature on an orbital shaker.

After incubation, the plate was washed five times with wash buffer and 200µl of Ellman's reagent (reconstituted immediately before use) was added into each well. The plate was then covered again with plastic film and left for 60-90 min. This assay is typically complete when B_0 wells have acquired ≥ 0.3 absorbance unit (A.U). The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412nm. The intensity of this colour, determined spectrophotometrically, is proportional to the amount of PGD_2 tracer bound to the well, which is inversely proportional to the amount of free PGD_2 present in the well during the incubation.

2.5.5 β-hexosaminidase assay

10 µl of supernatant was mixed with 10 µl of β-hexosaminidase substrate (4-nitrophenyl N-acetyl-β-d-glucosaminide) solution adjusted to pH = 4.5 with citrate buffer, and incubated for 1 h at 37 °C. 250 µl of 0.1 M of Na_2CO_3/$NaHCO_3$ solution was added, and absorbance at 405 nm was measured. Total release of mast cell contents was achieved by adding the cell lysate of unstimulated cells. Degranulation percentage was calculated using the formula: (β-Hex (supernatant)-A)/(β-Hex (total)-A) × 100, where A is the β-hexosaminidase released from unstimulated cells.

2.6 INVERTED LIGHT MICROSCOPY

CDMCs were plated at a density of 10^5 cells per well. The cells were stimulated with 5, 10 and 20 µg/ml of compound 48/80 for 10 min. In a separate group, the cells were pre-treated with 10 nM of nedocromil for 5 min prior to the stimulation of 20 µg/ml of compound 48/80. The morphology of these cells was compared to that of unstimulated cells. The cells were then analysed and micrographs were taken using the Inverted light microscope (Nikon) at 40x magnification. The total cells and degranulated cells were counted in a blinded manner for each treatment groups (n=3) and the percentage of CDMCs degranulation was calculated.

2.7 IMMUNOFLUORESCENT STAINING

2.7.1 Confocal microscopy studies to visualise Anx-A1 distribution in U937 cells

U937 cells were plated at density of 10^5 cells per chamber in microscope chamber slides. PMA (10ng/ml) was added to the cells for 24 h. Cells were treated with anti-allergic drugs (10nM) on their own or in combination with dexamethasone (2nM) respectively for 5 min. Upon treatment, cells were washed with PBC once and fixed with 2% paraformaldehyde (PFA) in 0.1M PBS for 10 min on ice. Cells were washed again in 0.1M PBS and Phalloidin Red (Alexa Fluor 546) was added into the cells at 1:1000 dilution for 10 min. Cells were then permeabilised with 0.1% Triton X-100 in PBS for 5 min. Non-specific secondary antibody binding site were blocked by incubation in 10% FBS in PBS for 30 min.

Primary antibody (Anx-A1, Zymed, Invitrogen) was diluted (1:1000) with 1% FBS in PBS and added into the chamber slides and left on the orbital shaker for 1 h at room temperature. Cells were then washed twice with PBS containing 1% FBS and incubated in the dark with fluorescent-labelled secondary antibody (Alexa Fluor 488) at 1:500 dilution.

Cells were washed twice in PBS and nuclei counterstained with 100ng/ml DAPI in ddH_2O for 5 min. Slides were washed with ddH_2O and air-dried. Cover slips were carefully mounted onto the slides using the Prolong Gold Mountant. Slides were then analysed and micrographs were taken using

the confocal microscope (Leica) at 63x magnification.

2.7.2 Confocal microscopy to observe the distribution of PKC, tryptase, and Anx-A1 in cord blood derived mast cells (CDMCs).

CDMCs were plated at a density of $2x10^5$ cells in a 1.5ml Eppendorf tube. Cells were treated with the stipulated drugs for 5 min prior to stimulation with compound 48/80. 2% PFA in 0.1M PBS was added into the Eppendorf for 10 min on ice to fix the cells. Since the CDMCs are non-adherent cells, each immunostaining steps were performed in 1.5ml Eppendorf tube and centrifuged at 1000g for 5 min between each methodological step and the supernatant was aspirated carefully with a vacuum without disrupting the pellet. After fixation, the cells were washed with 0.1M PBS. Cells were then permeabilised with 0.1% Triton X-100 in PBS for 5 min. Non-specific secondary antibody binding site were blocked by incubation of 10% FBS in PBS for 30 min.

Primary antibody (refer to Table 2.4) was diluted with 1% FBS in PBS and left to incubate at 4°C overnight. The cells were washed twice with 1% FBS in PBS and incubated with fluorescent-labelled secondary antibody (refer to Table 2.4) for 1 h at room temperature.

The cells were then washed with PBS and stained with 100ng/ml 4',6-diamidino-2-phenylindole (DAPI; Invitrogen)) in ddH_2O for 5 min. The cells were washed with 30µl ddH_2O and dropped onto the slide using a pipette.

Cover slips were carefully mounted onto the slides using the Prolong Gold Mountant (Invitrogen). Slides were then analysed and micrographs were taken using the confocal microscope (Leica) at 63x magnification.

Primary Ab	Host	Dilution	Type	Source	Secondary Ab
Anx-A1 (1B)	mouse	1:1000 (10µg/ml)	Monoclonal	In-house (Clone 1B)	Alexa Fluor 488 (green)
Anx-A1	rabbit	1:1000 (10µg/ml)	Polyclonal	Invitrogen (71-3400)	Alexa Fluor 546 (red)
Tryptase	mouse	1:100 (100µg/ml)	Monoclonal	Abcam (ab2378)	Alexa Fluor 488 (green)
Total PKC	rabbit	1:50 (200µg/ml)	Polyclonal	Cell Signalling Technology (2056)	Alexa Fluor 546 (red)

Table 2.4: Primary and secondary antibodies used for confocal microscopy.

2.8 STATISTICAL ANALYSES

Before performing any relevant tests, the samples were analysed for normality. All values are presented as mean ± SEM pooled data from 3 independent experiments. Data were analysed using a one-way analysis of variance (ANOVA), followed by a Bonferonni post hoc test unless otherwise stated. All statistical tests were performed with GraphPad Prism version 4 for Mac (GraphPad Software, San Diego, California, USA, www.graphpad.com). $P < 0.05$ was considered to be statistically significant.

3. RESULTS

3.1 THE EFFECTS OF ANTI-ALLERGIC DRUGS ON ANX-A1 PHOSPHORYLATION.

3.1.1 The effect of dexamethasone on Anx-A1 phosphorylation in U937 cells.

Anx-A1, which is mainly found in the cytoplasm, translocates rapidly to the cell surface upon treatment with glucocorticoid and cromones (Solito *et al.*, 1994; Yazid *et al.*, 2009) and is secreted into the extracellular milieu where it exert its anti-inflammatory activities. This rapid GR-dependent non-genomic event is preceded by the phosphorylation of Anx-A1 on a specific amino acid residue Ser^{27} (Solito *et al.*, 2003c; Solito *et al.*, 2006b). In preliminary experiments, no effect of dexamethasone on Anx-A1 phosphorylation was observed in the pro-monocytic cell line U937 in the absence of the PMA pre-treatment (Figure 3.1), because the cells need to acquire monocytic properties to become sensitive to the action of GC. However, upon 'priming' for 24 h with phorbol-12-myristate-13-acetate (PMA), the U937 cells were responsive towards the GC treatment (Solito *et al.*, 1991a).

Since 5 min pre-treatment with GC and cromones produced the maximal effects on Ser^{27}-Anx-A1 phosphorylation in U937 cells differentiated with 10ng/ml of PMA for 24 h (Yazid *et al.*, 2009), this time point was chosen for all the experiments performed on U937 cells, in which the effects of anti-allergic drugs (0 - 500nM) were determined. Dexamethasone (0 - 200nM)

increases Ser^{27}-Anx-A1 phosphorylation in a concentration dependent-manner in U937 cells (Figure 3.2).

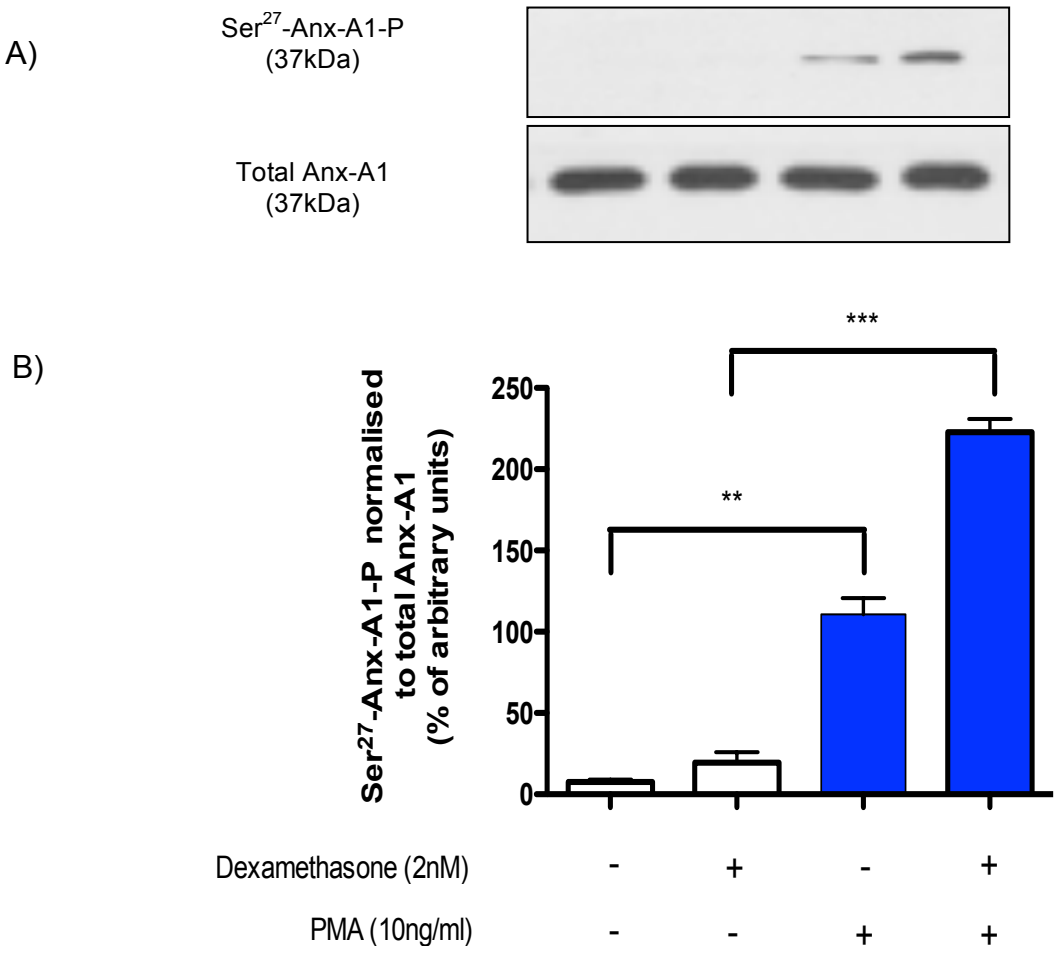


Figure 3.1: Ser^{27}-Anx-A1-P detection in U937 cells with dexamethasone (2 nM) in the presence or absence of PMA (10ng/ml).

(A) Determination of Ser^{27}-Anx-A1-P by Western blot. Cells were treated with dexamethasone (2nM), with or without PMA (10ng/ml) and lysed with 100µl of lysis buffer. 30µg of sample were loaded after protein concentrations and were assessed by Bradford Assay. Equal loading was confirmed by total Anx-A1. (B) Densitometry values of 3 independent experiments were expressed as percentage of arbitrary units of Ser^{27}-Anx-A1-P expressed in whole cell lysate. Data are expressed as mean ± SEM; (** $p<0.01$ and *** $p<0.001$).

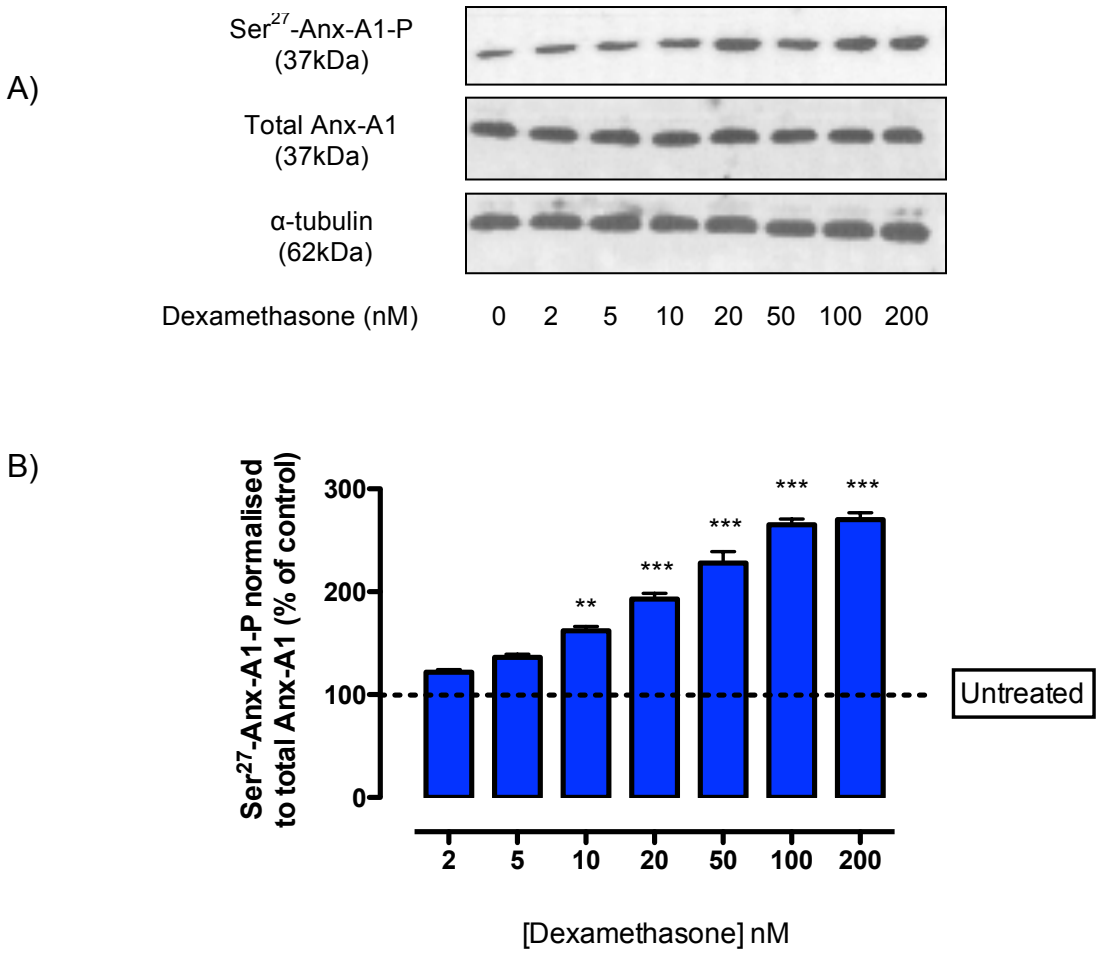


Figure 3.2: Dexamethasone increases Ser^{27}-Anx-A1 phosphorylation in U937 cells in a concentration-dependent manner.

(A) Detection of Ser^{27}-Anx-A1-P was determined by Western blot in U937 cells treated for 5 min with dexamethasone at various concentrations (0 - 200nM). (B) Densitometry values from 3 independent experiments were expressed as mean percentage of control of the Ser^{27}-Anx-A1-P normalised to total Anx-A1 expressed in the whole cell lysate. Data are expressed as mean ± SEM; **p<0.01 and ***p<0.001.

3.1.2 The ability of anti-allergic drugs to promote Ser^{27}-Anx-A1 phosphorylation in a concentration-dependent manner.

Three H_1 antagonist drugs, promethazine, pheniramine and antazoline were tested in this system. These drugs, which are known as anti-histamines, are commonly used in the treatment of allergic conditions. Immunoblotting techniques was utilised to determine the concentration-response of these drugs to promote the phosphorylation of Anx-A1 in U937 cells. Figure 3.3 show that antazoline produced a weak effect from 2 - 50nM (2 fold change from control) and subsequently decreased below the basal level with increasing concentrations. Whereas, promethazine and pheniramine shares the same trend, whereby, from 2 - 100nM, these drugs did not promote phosphorylation of Anx-A1 at Ser^{27} residue. However, from 100 - 500nM, these drugs produced a weak concentration-dependent increase of Ser^{27}-Anx-A1-P, with maximal phosphorylation observed at 500nM with < 2-fold change.

'Mast cell stabilising' drugs (nedocromil, cromoglycate and pemirolast) were used in this system to study the ability of these drugs to enhance the phosphorylation of Anx-A1. Based on the blots (Figure 3.4), all these drugs promoted the phosphorylation of Anx-A1 across increasing concentrations (0 - 500nM). Among the three drugs, nedocromil has the highest efficacy by producing the maximal effect at 500nM (> 3-fold change), whilst cromoglycate and pemirolast induces maximal effects of only 2.5-fold change.

'Dual-action' drugs have the capacity to antagonise the H_1 receptor and 'stabilise' the mast cells upon allergic challenge. Ketotifen, epinastine and olopatidine were tested on U937 cells and their ability to promote Ser^{27}-Anx-A1-P was determined by Western blotting. Figure 3.5 shows that epinastine and ketotifen alone greatly potentiated the Anx-A1 phosphorylation in a concentration-dependent fashion. Epinastine and ketotifen induce > 5-fold change and 4 fold-change respectively at the highest concentration. Whereas, olopatidine is not as efficacious as the other two drugs as it only promoted Anx-A1 phosphorylation by 2-fold at 500nM.

To conclude this part, these anti-allergic drugs could be categorised on their ability to promote the phosphorylation of Anx-A1 at Ser^{27} residue (Table 3.1). H_1 antagonist drugs only weakly increase phosphorylation of Anx-A1 (< 2-fold). Drugs that moderately (< 2.5-fold) induce Anx-A1 phosphorylation are pemirolast, cromoglycate and olopatidine. Whereas, Anx-A1 is greatly (> 3-fold) phosphorylated in the presence of nedocromil, ketotifen and epinastine.

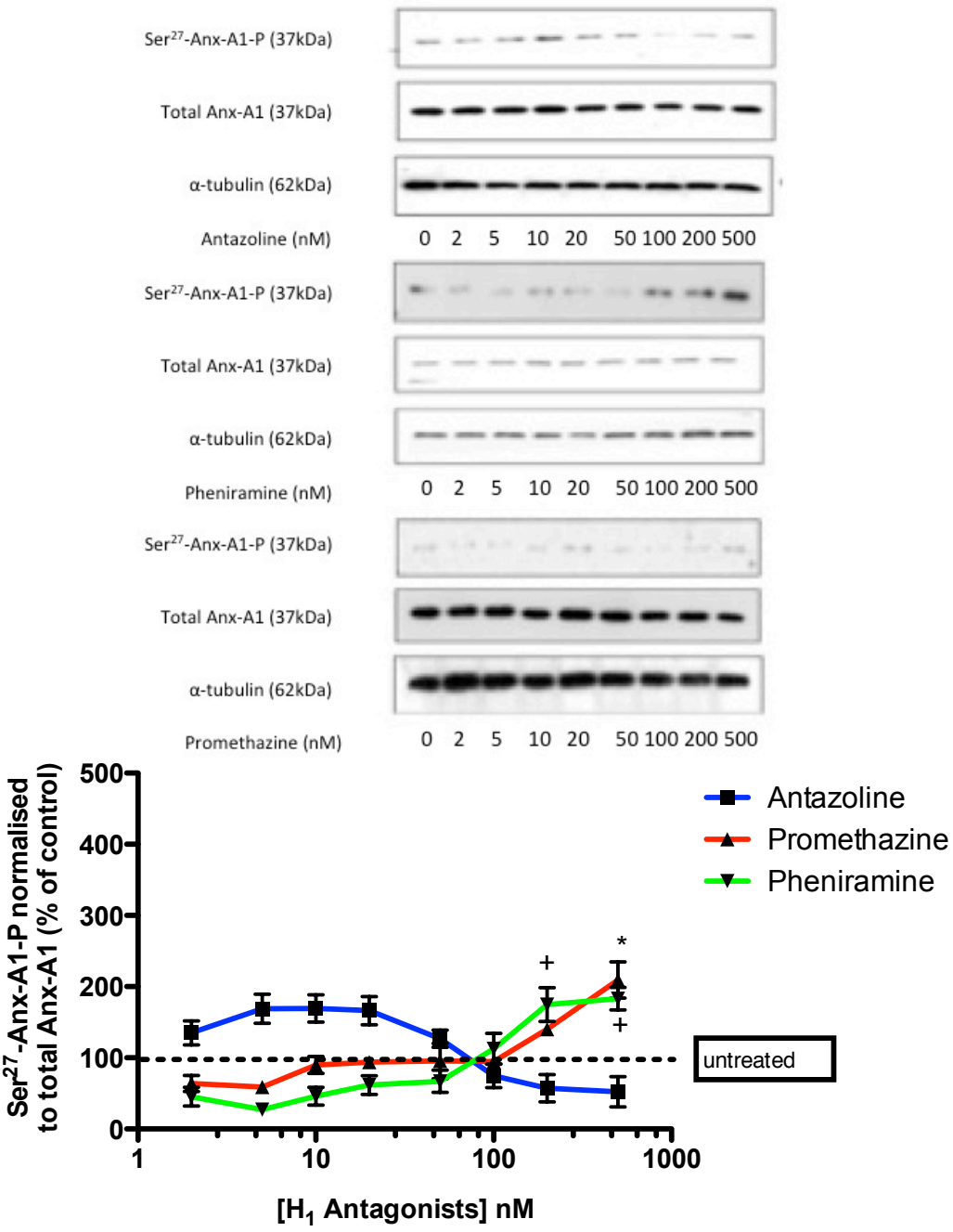


Figure 3.3: H_1 antagonist drugs exert weak Ser^{27}-Anx-A1 phosphorylation in U937 cells.

U937 cells were treated with increasing concentrations (2 - 500nM) of H_1 antagonist drugs for 5 min and processed for cell lysis protocol and protein assay. 30µg of sample was loaded into each well and Anx-A1 phosphorylation was detected by Western blots. Data are expressed as mean ± SEM and is a representative of 3

independent experiments. (Promethazine: * p<0.05 *vs* untreated and pheniramine: $^{+}$ p<0.05 *vs* untreated).

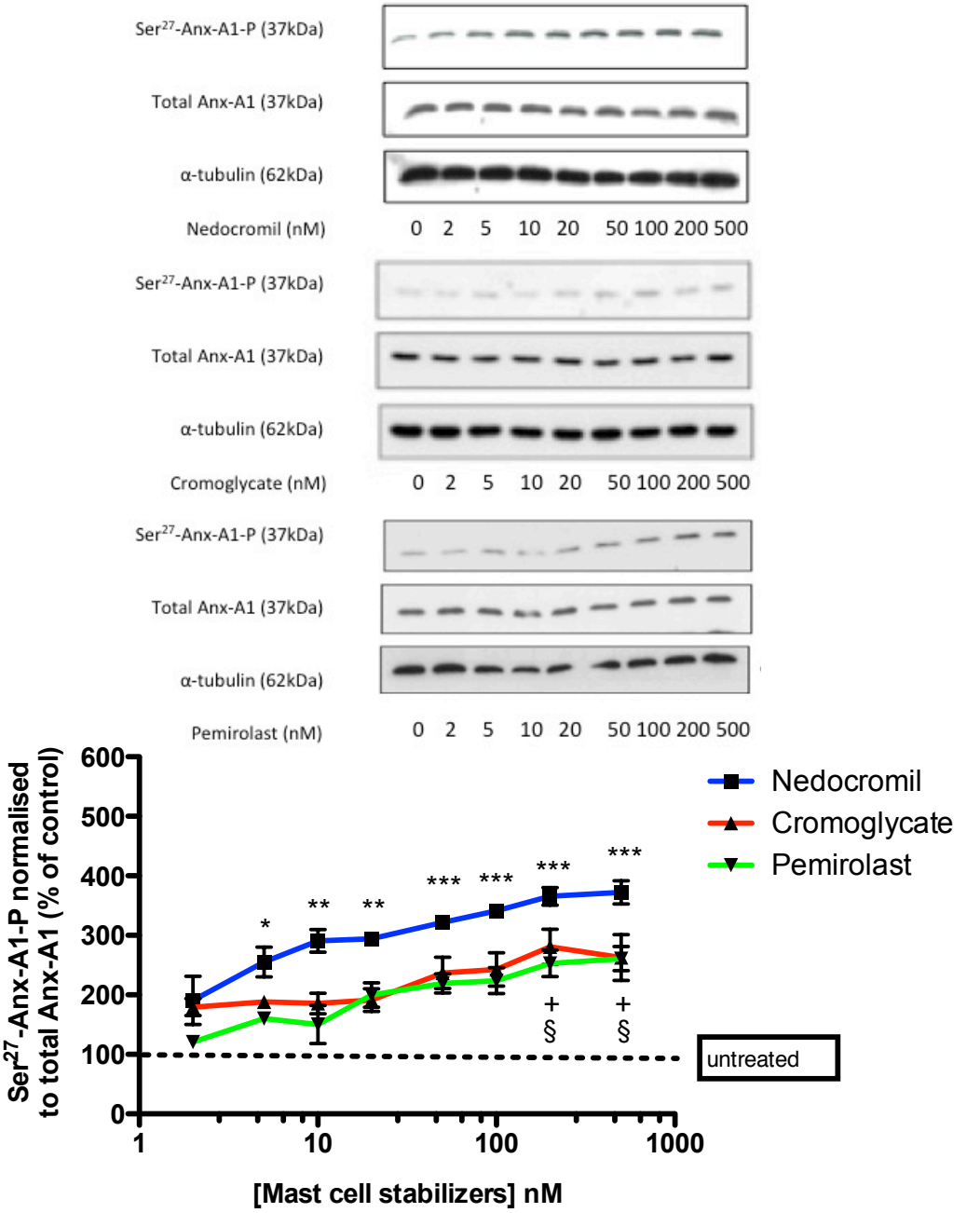


Figure 3.4: Individual treatment of cromoglycate and pemirolast exert moderate Anx-A1 phosphorylation, however nedocromil induce strong signal of Anx-A1 phosphorylation in U937 cells.

Aliquots of $2x10^5$ U937 cells were cultured as described and incubated for 5 min with escalating concentrations of nedocromil, cromoglycate and pemirolast (2 - 500nM). The cell lysate were prepared for Western blotting to assess the intracellular concentrations of Ser^{27}-Anx-A1-P. Data are expressed as mean ± SEM and is a representative of 3 independent experiments. (Nedocromil: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, cromoglycate: $^{+}$ $p<0.05$, pemirolast: § $p<0.05$ *vs* untreated).

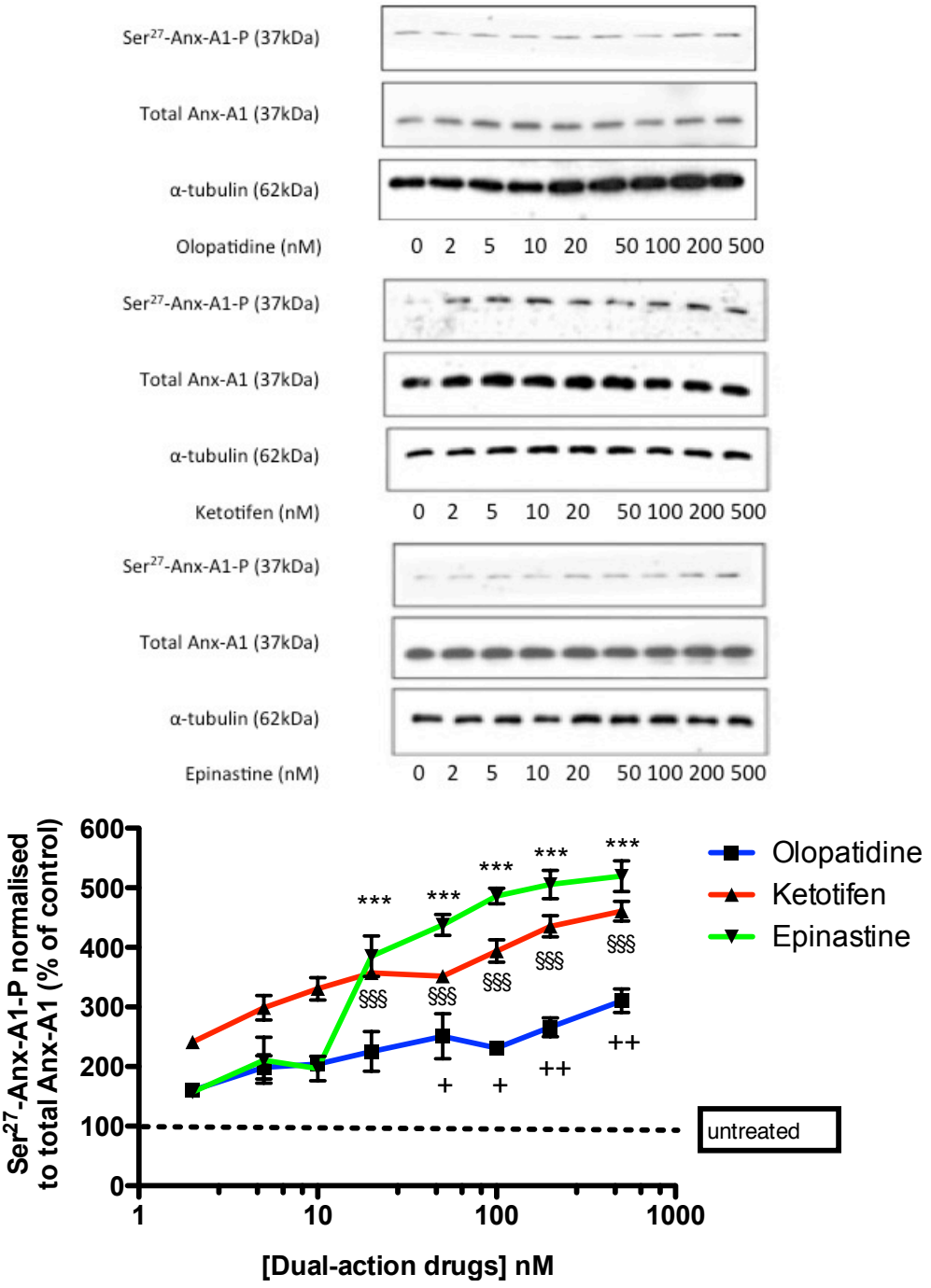


Figure 3.5: 'Dual-action' drugs increases Ser^{27}-Anx-A1 phosphorylation in a concentration-dependent manner in U937 cells.

The cell lysate were prepared for Western blotting to assess the intracellular concentrations of Ser^{27}-Anx-A1-P upon treatement with olopatidine, ketotifen and epinastine (2 - 500nM). Based on the densitometry analysis, ketotifen and epinastine

are able to promote strong Anx-A1 phosphorylation; meanwhile olopatidine only moderately increases phosphorylation of Anx-A1. Data are expressed as mean ± SEM and is a representative of 3 independent experiments. (Epinastine: *** p<0.001, ketotifen: §§§ p<0.001, olopatidine: + p<0.05, ++ p<0.01 *vs* untreated).

Anx-A1 phosphorylation	Weak (< 2 fold increase)	Moderate (< 2.5 fold increase)	Very active (> 3 fold increase)
H_1 antagonists only	Promethazine Pheniramine Antazoline		
'Mast cell stabilisers' only		Pemirolast Cromoglycate	Nedocromil
'Dual-action' drugs		Olopatidine	Ketotifen Epinastine

Table 3.1: Fold change is based on densitometry analysis of Ser^{27}-Anx-A1-P in U937 cells treated with anti-allergic drugs.

3.1.3 Effects of anti-allergic drugs alone or in combination with dexamethasone (2nM) on the distribution and release of Anx-A1 in U937 cells.

Anx-A1, mainly localised in the cytosol, is exported to the cell membrane when cells are briefly exposed to dexamethasone (Solito *et al.*, 2003c). Once at the cell membrane, Anx-A1 is secreted either in an autocrine or paracrine manner to exert its anti-inflammatory properties. A previous study has shown that nedocromil in combination with dexamethasone caused an increased release of Anx-A1 in U937 cells (Yazid *et al.*, 2009). Therefore, in this study, the distribution of total Anx-A1 protein upon treatment with anti-allergic drugs alone or in combination with dexamethasone was investigated. Supernatants from U937 cells treated with the drugs were collected and assessed for total Anx-A1 protein by Western blotting. Since this technique is semi-quantitative and Anx-A1 expression measured outside of the cell cannot be normalised to a standard protein, it is difficult to characterise the shape of the concentration-response curve across increasing concentrations. From these blots (data not shown), only the consecutive release of total Anx-A1 upon treatment was observed. Hence, the Western blot analysis was corroborated by assessing the distribution and release of Anx-A1 by confocal microscopy imaging technique, whereby cells were stained with DAPI to view the nucleus (blue), Phalloidin Red Alexa Fluor® 546 labelling specifically the membrane phospholipids (red) and an antibody against Anx-A1 (Zymed,

Invitrogen) which labels total Anx-A1 (green).

The effect of the H_1 antagonists on the intracellular movement of Anx-A1 at microscopic level was investigated. Positive controls are shown in Figure 3.6 (A). Cells treated with H_1 antagonist drugs alone exhibit a very weak Anx-A1 signal (green), but in the presence of dexamethasone, distinct co-localisation, which is evident from the yellow signal, was observed, followed by the release of Anx-A1 (Figure 3.6).

Confocal images show that all the three 'mast cell stabilising' drugs were able to release Anx-A1, in that, cells treated with these drugs (10nM) alone exhibit distinct co-localisation with the membrane and release of Anx-A1. However, these effects are greatly enhanced in the presence of dexamethasone at 2 nM (Figure 3.7).

Figure 3.8 shows that epinastine, ketotifen and olopatidine provoke strong release of Anx-A1 on their own with the evidence of membrane blebbing; however, dexamethasone does not produce any further effect when combined with these drugs.

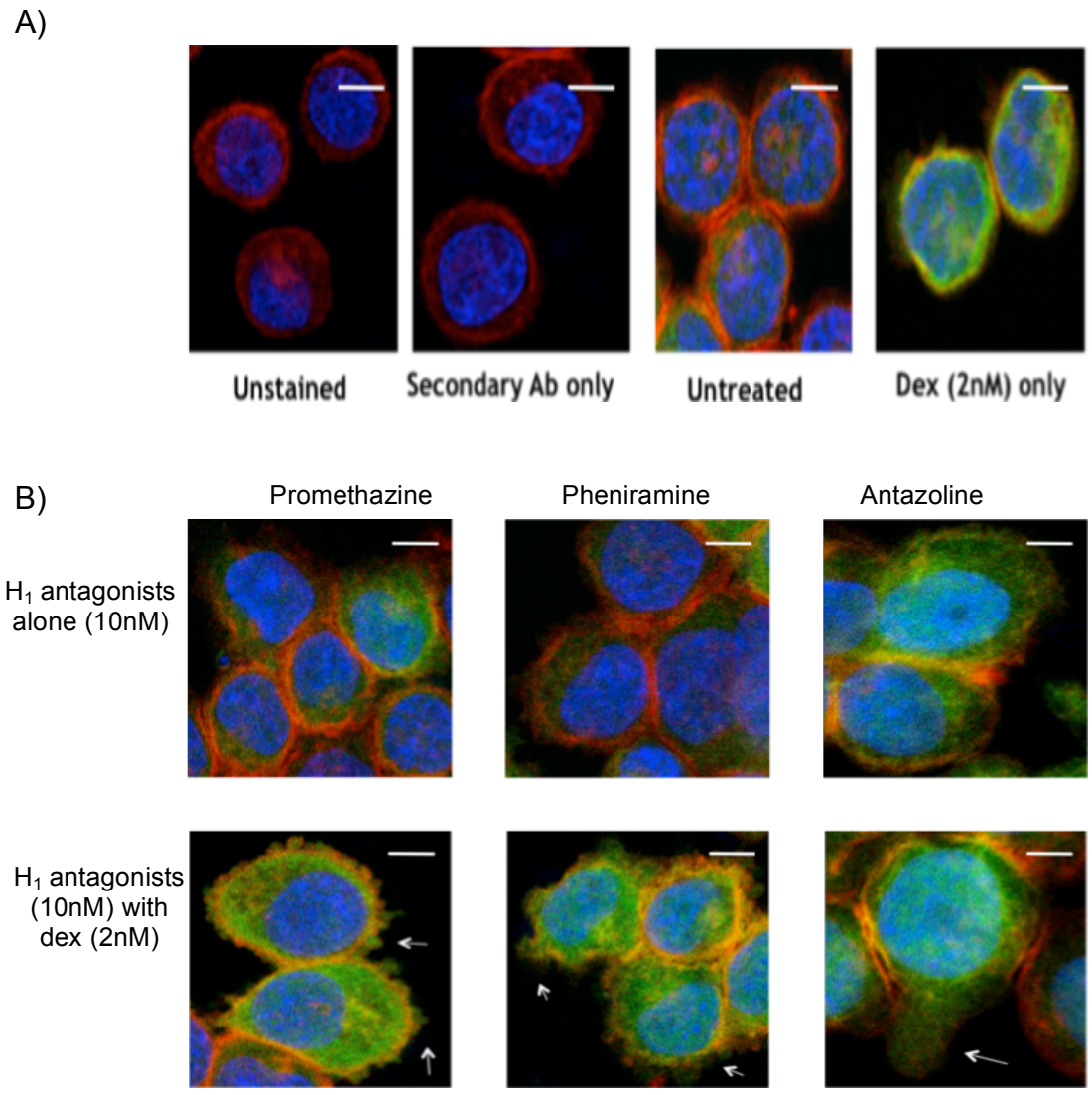


Figure 3.6: H_1 antagonist drugs promote weak Anx-A1 signal.

Panel A shows positive control cells. U937 cells were stained with DAPI (blue), an antibody against total Anx-A1 (green) and phalloidin (red). (B) Confocal micrographs of U937 cells treated for 5 min with H_1 antagonist drugs alone or in combination with dexamethasone (2nM). These drugs provoke very weak Anx-A1 release on their own but in the presence of dexamethasone, there is a striking degree of co-localisation between Anx-A1 and phalloidin red and evidence of membrane 'blebbing' was observed. Pictures were taken at x63 magnification. Scale bars indicate 10μm. These images are representative of 3 independent experiments.

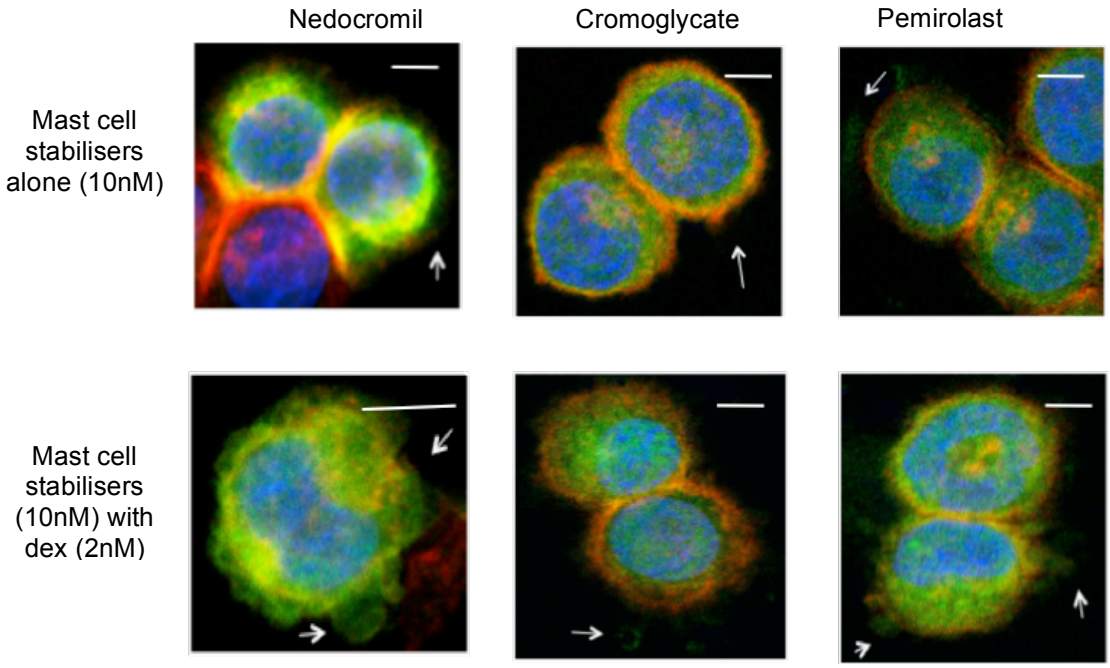


Figure 3.7: Moderate Anx-A1 secretion was seen after treatment with 'mast cell stabilising' drugs but is potentiated by dexamethasone.

Confocal microscopy shows that in nedocromil treated cells, Anx-A1 is distributed at the plasma membrane, but in the presence of dexamethasone, prominent Anx-A1 secretion was observed. Anx-A1 was found to be distributed mainly in the cytoplasm of U937 cells treated with both cromoglycate and pemirolast. However, when dexamethasone was administered to the cells, distinct Anx-A1 secretion was observed. Pictures were taken at x63 magnification. Scale bars indicate 10μm. These images are representative of 3 independent experiments.

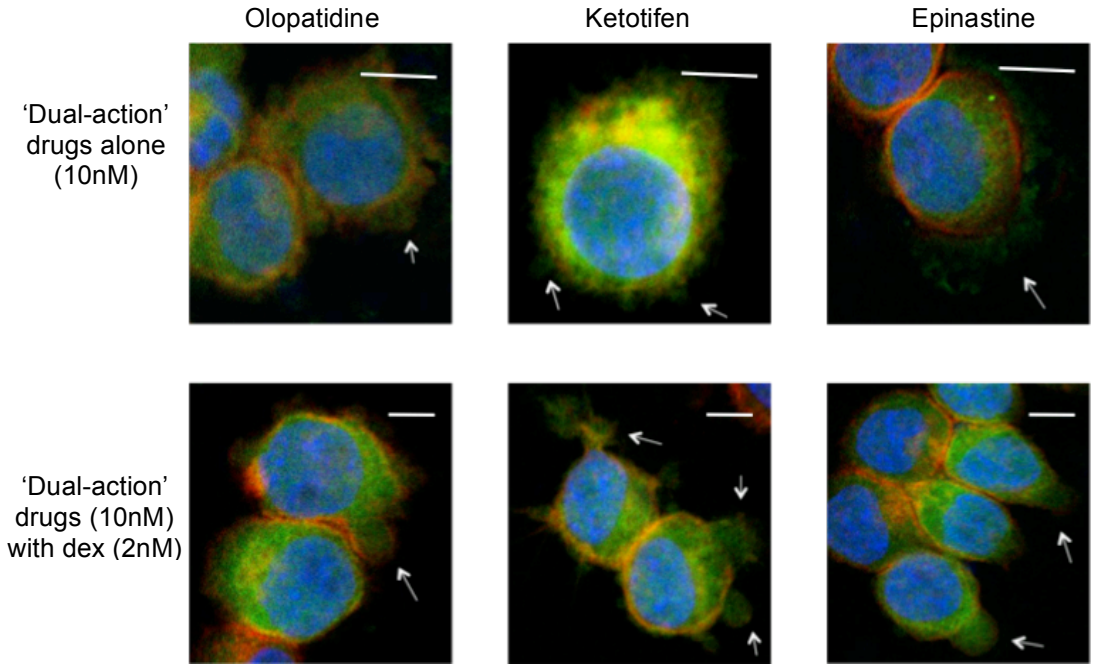


Figure 3.8: Anx-A1 secretion was strongly induced by 'dual-action' drugs.

Confocal micrographs show that dual-action anti-histamine drugs displayed prominent membrane protrusions (white arrow) and released Anx-A1 in a concentration-dependent fashion. Similar morphological changes were observed when 'dual-action' drugs were combined with dexamethasone. Pictures were taken at x63 magnification. Scale bars indicate 10μm. These images are representative of 3 independent experiments.

3.1.4 Nedocromil potentiates the inhibition of thromboxane B_2 (TxB_2) release in the presence of dexamethasone in U937 cells.

The ability of GCs to inhibit eicosanoid synthesis in many systems has been shown to depend on the release of Anx-A1 (Errasfa *et al.*, 1989; Croxtall *et al.*, 2003). TxB_2 was measured because this mediator could be used as an index of eicosanoid production. U937 cells were used because TxB_2 is generated in abundance by these cells compared to other eicosanoid mediators (Solito *et al.*, 1991a). Figure 3.9 shows that increasing concentration of nedocromil (2 - 100nM) produces a modest suppression of TxB_2 release into the supernatant of U937 cells with maximal inhibition of 18.2 ± 2.8% ($p<0.05$). Pre-treatment of dexamethasone alone (2nM) for 5 min, only produced 3.9 ± 0.5% inhibition. However in the presence of dexamethasone at 2nM, the actions of nedocromil was greatly potentiated across increasing concentrations, such that TxB_2 was significantly ($p<0.001$) suppressed even further by 63.9 ± 1.8% at 100nM of nedocromil.

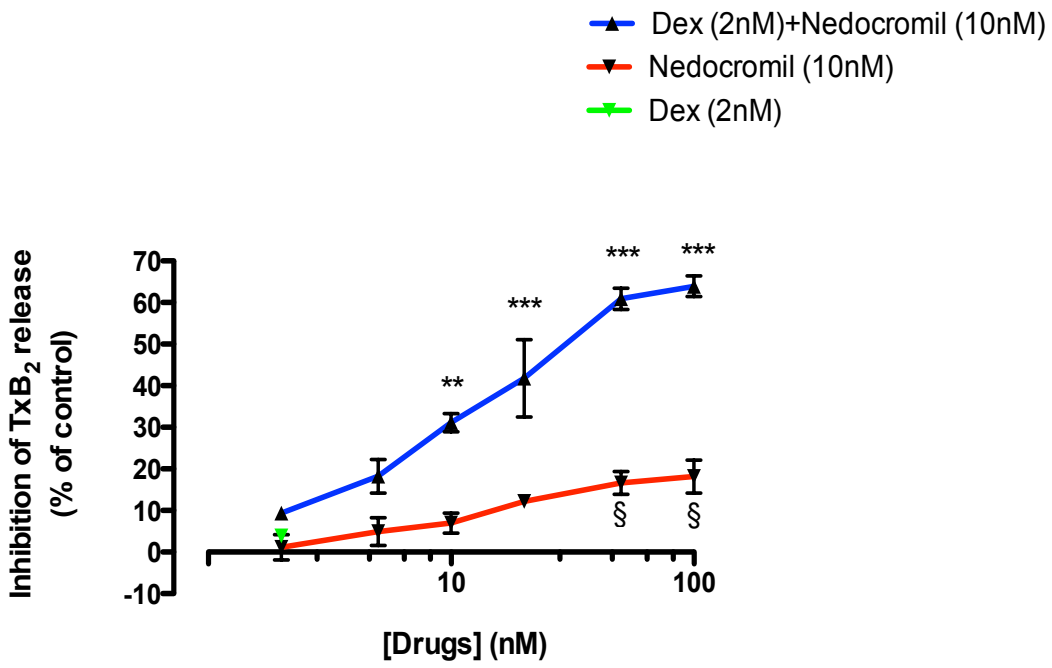


Figure 3.9: Measurement by ELISA assay of TxB_2 release shows that nedocromil inhibits TxB_2 released by U937 cells in a concentration-dependent manner in the presence of dexamethasone.

When dexamethasone (2nM) is combined to the increasing concentrations of nedocromil for 5 min, a concentration-dependent potentiation of the inhibitory effects of TxB_2 release was observed. Data are expressed as mean ± SEM and is a representative of 3 independent experiments. **p<0.01 and ***p<0.001 and § p<0.05 relative to untreated.

3.2 ANTI-ALLERGIC DRUGS STIMULATE ANX-A1 THROUGH A PKC-DEPENDENT MECHANISM IN CDMCS.

3.2.1 Anx-A1 phosphorylation is abolished by an inhibitor of PKC.

Even though much work has been done on the effect of the cromoglycate-like drugs such as nedocromil on Anx-A1 and PKC phosphorylation in U937 cells (Yazid *et al.*, 2009; Yazid *et al.*, 2011), the main clinical target of these drugs are the mast cells. Thus, this led to the investigation on the interaction between the anti-allergic drugs, Anx-A1 and PKC in the human mast cells.

In a human folliculostellate cell line, the phosphorylation of Anx-A1 produced by dexamethasone is the result of the activation of protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and phosphatydilinositol 3-kinase (PI3K) (Solito *et al.*, 2003c). Yazid *et al* has shown that PKC activation is responsible for the phosphorylation of Anx-A1 in the U937 cells (Yazid *et al.*, 2009). Since kinase activation may be dependent on cell type, the importance of PKC in the cord-blood derived mast cells (CDMCs) was assessed by using a PKC inhibitor (Gö 6983). The CDMCs were treated with 10nM of nedocromil for 5 min, with or without the presence of PKC inhibitor for 30 min (10μM).

Figure 3.10 shows that nedocromil-induced Anx-A1 phosphorylation (136.3 ± 1.1%) is blocked significantly by the addition of PKC inhibitor (43.5 ± 11.9%; *$p<0.05$ *vs* nedocromil alone). Even though there is a slight increase

of Anx-A1 phosphorylation in the presence of nedocromil and PKC inhibitor as compared to cells incubated with PKC inhibitor alone, the statistical analysis revealed that these differences were not significant. It might imply that possibly a small residual activity of PKC was not completely abolished by the inhibitor. However, previous study has shown that the increase in Anx-A1 phosphorylation by nedocromil was a result of PKC activation in U937 cells (Yazid *et al.*, 2009).

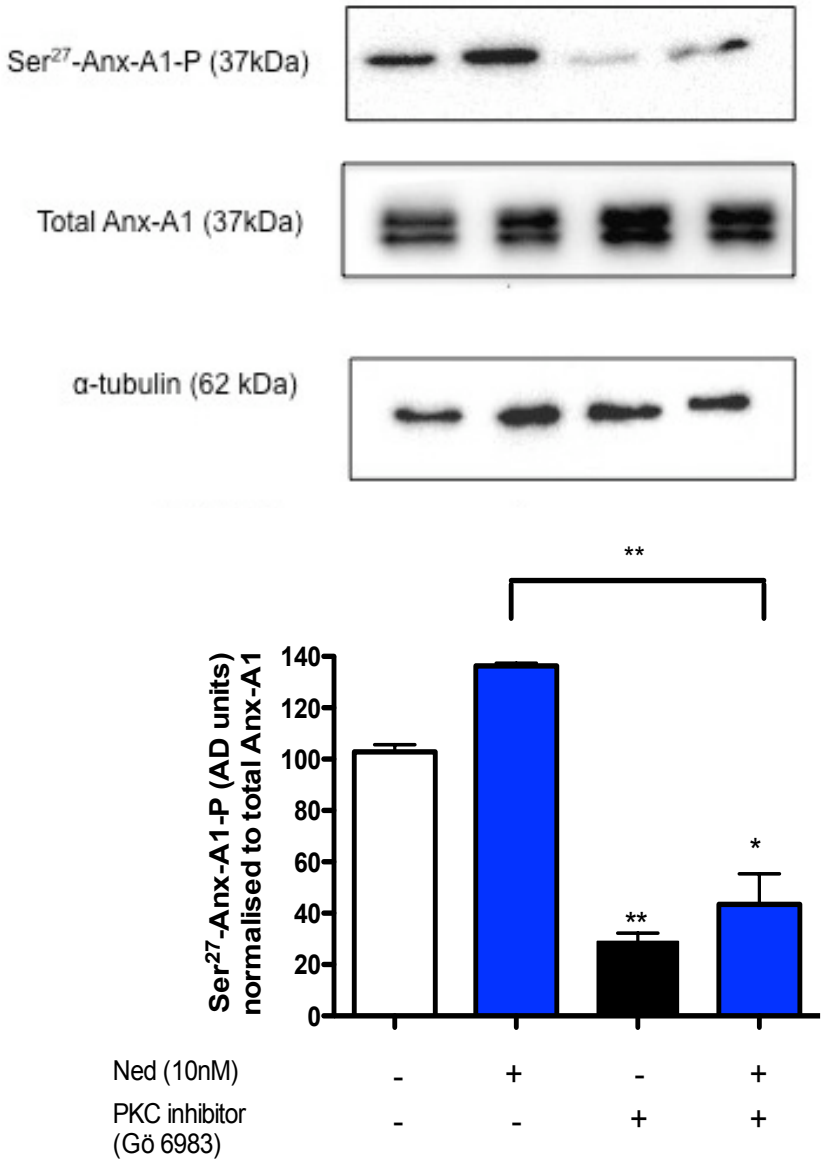


Figure 3.10: The increase in Ser^{27}-Anx-A1-P by nedocromil is a result of PKC activation in CDMCs.

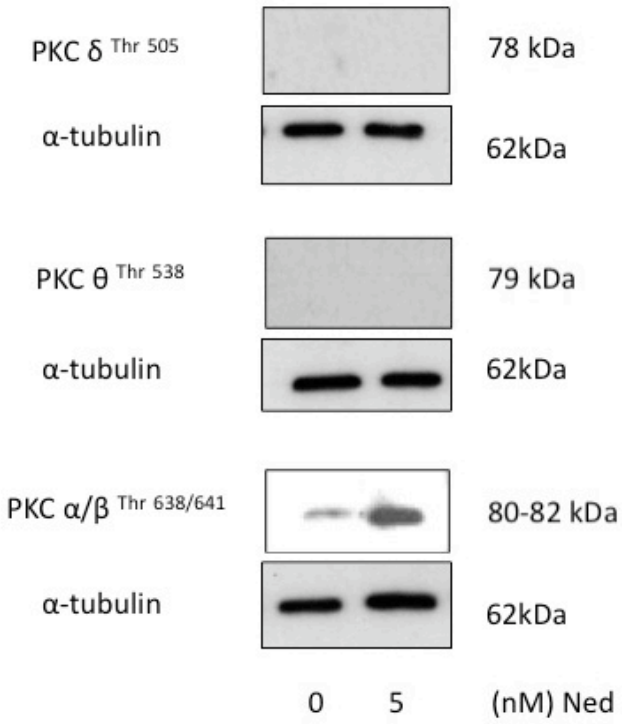
CDMCs were incubated with PKC inhibitor (Gö 6983; 10μM) for 30 min prior to the 5 min pre-treatment of nedocromil (10nM). Densitometry analysis shows that nedocromil was not able to induce Anx-A1 phosphorylation in the presence of PKC inhibitor in CDMCs. Representative blots of 3 independent experiments. Data are

expressed as mean ± SEM; *p<0.05 and **p<0.01 relative to untreated or nedocromil alone.

3.2.2 Western blotting detection of specific PKC isoforms.

Figure 3.10 show that phosphorylation of Anx-A1 in CDMCs is dependent on PKC phosphorylation but not which isoform. Hence, a panel of anti-phospho PKC isoforms antibodies were analysed using Western blot. Given that the molecular weight of the PKC using pan-specific PKC antibody was 78 - 85kDa, it suggested that the relevant isoforms were PKCδ (78kDa), PKCθ (79kDa) or PKCαβ (80 - 82kDa). Figure 3.11 shows that that PKCδ and PKCθ were not detected in CDMCs treated with 0 and 5 nM of nedocromil. However, the specific $PKC_{\alpha/\beta}^{Thr\ 638\text{-}641}$ antibody showed good reactivity with a band that increased with 5nM of nedocromil over a period of 5 min. Therefore it could be concluded that the main kinase responsible in this system is probably PKCα/β (80-82kDa).

A)



B)

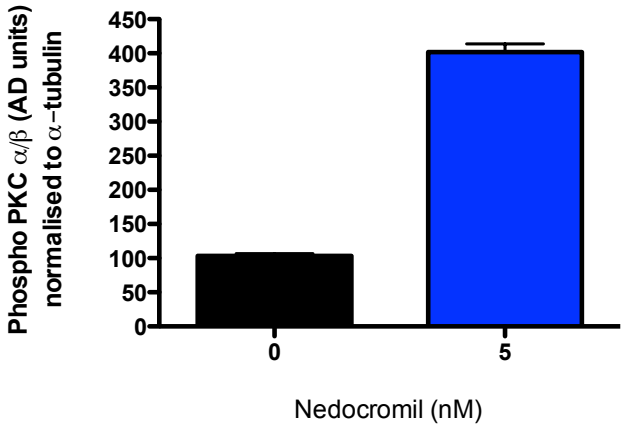


Figure 3.11: $PKC_{\alpha/\beta}^{Thr\ 638\text{-}641}$ isoform is responsible for nedocromil-induced Ser^{27}-Anx-A1-P in CDMCs.

A) Analysis of the potential PKC isoform activated by nedocromil at 0 and 5nM was performed by Western blot. Blots were probed with different anti-sera specific for each isoform. Activated $PKC_{\alpha/\beta}^{Thr\ 638\text{-}641}$ was the only isoform to show a strong signal in response to nedocromil treatment in human mast cells. Representative blots of 3

independent experiments. B) Densitometry analysis was performed for PKC $_{\alpha/\beta}^{\text{Thr 638-641}}$ normalised to α-tubulin.

3.2.3 The effects of anti-allergic drugs on Anx-A1 phosphorylation in CDMCs.

Working with collaborators, our group has reported the anti-allergic action of the Anx-A1 derived peptide ac2-26 in a rat pleurisy model (Bandeira-Melo *et al.*, 2005). Thus, the effect of the anti-allergic drugs such as promethazine, nedocromil and ketotifen, on Anx-A1 and PKC phosphorylation in the cord-blood derived mast cells (CDMCs) was assessed.

Firstly, the optimal time point for PKC-P and Ser^{27}-Anx-A1-P in CDMCs treated with nedocromil was established. Figure 3.12 shows that phosphorylation of Anx-A1 in response to nedocromil treatment was already maximal at 5 min (~ 2 fold) and declined within 15 min (27.4 ± 2.8% *vs* 100% control). The same trend was observed when the blots were probed for PKC phosphorylation; at 5 min the optimal effect was observed (~ 4 fold). Therefore 5 min was chosen as the optimal time point for the anti-allergic drug treatment in CDMCs to study PKC and Anx-A1 phosphorylation.

The concentration-dependency of Anx-A1 and PKC phosphorylation in CDMC cell lysates induced by anti-allergic drugs following 5 min incubation was established by Western blot. In the untreated lane, small amounts of phospho-PKC and phospho-Anx-A1 were detected in the lysate. This is a common finding as these cells are already partly ‘activated’ by the presence of the stem cell factor (SCF) in the medium.

A panel (promethazine, nedocromil and ketotifen) of anti-allergic drugs (0 - 100nM) was compared in this experiment. Figure 3.13 show that increasing concentrations of promethazine did not significantly promote Anx-A1 phosphorylation across increasing concentrations. However, at concentrations from 5 - 10nM, promethazine significantly ($p<0.05$) increased PKC phosphorylation by 173.1 ± 15%. Based on Figure 3.14, nedocromil produces a maximal effect of Anx-A1 and PKC phosphorylation at 20nM (approximately 249.4 ± 19.7% and 331.0 ± 19.6% from control respectively). In contrast, beyond 20nM, no further effect is seen. Figure 3.15 show that increasing concentrations of ketotifen displays a 'bell-shaped' curve, with a maximal effect on Anx-A1 ($p<0.01$) and PKC ($p<0.001$) phosphorylation at 10nM (261.8 ± 9.4% and 304.8 ± 16.5% *vs* control) and thereafter a concentration-dependent decrease in the level of both Anx-A1 and PKC phosphorylation was observed. These results show that both nedocromil and ketotifen share similar ability to induce Anx-A1 and PKC phosphorylation, however promethazine did not significantly promote the phosphorylation of Anx-A1 and only weakly induced PKC phosphorylation. Based on the blots, it could be observed that Ser^{27}-Anx-A1 phosphorylation mirrors PKC phosphorylation in all the three drugs that were tested.

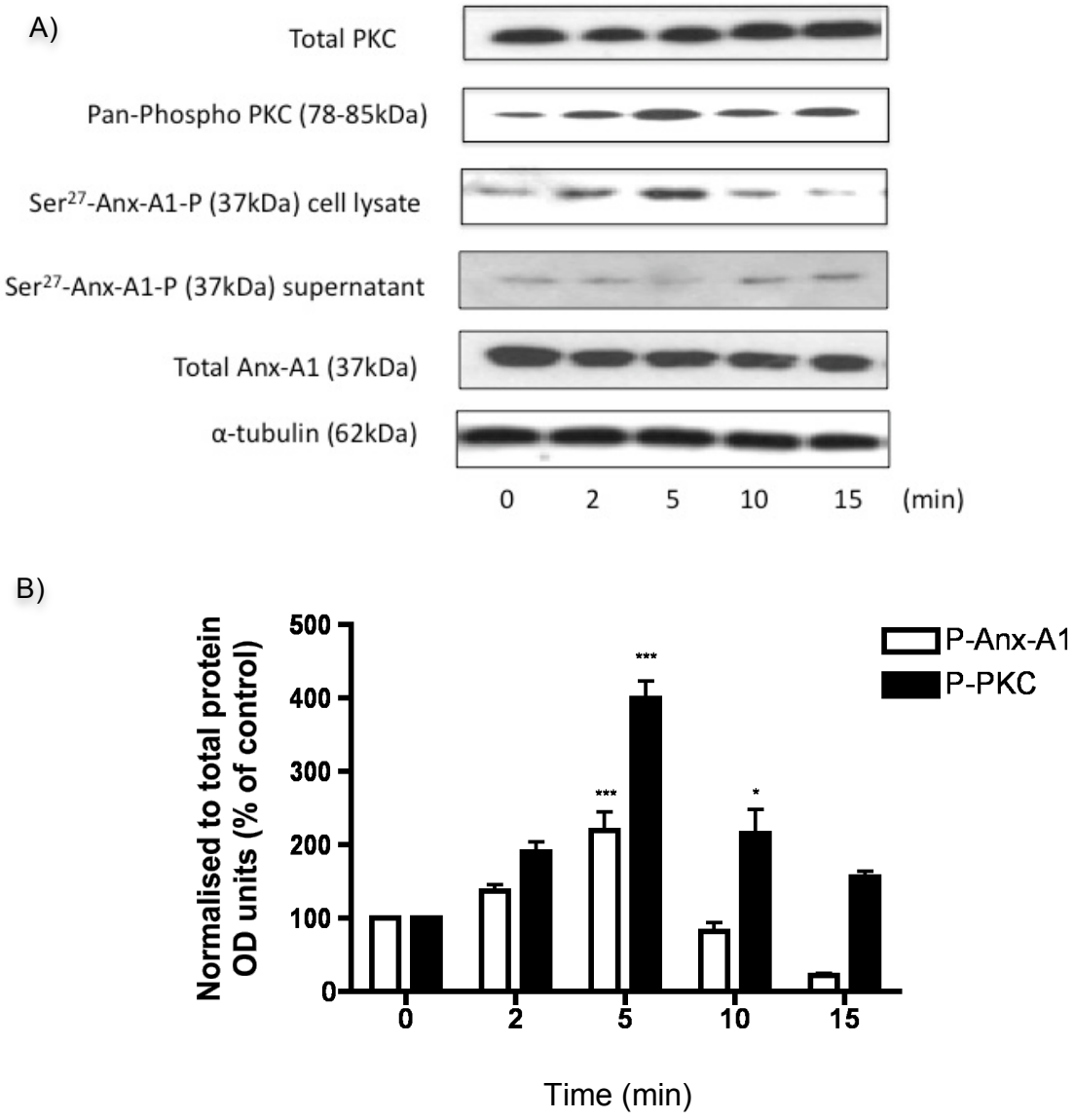


Figure 3.12: PKC-P and Ser^{27}-Anx-A1-P detection at 5 min in CDMCs treated with nedocromil (10nM).

(A) CDMCs were treated with nedocromil for periods of 0-15 min, processed for Western blotting and probed for PKC-P, total PKC, Ser^{27}-Anx-A1-P and total Anx-A1 antibodies. Equal loading was confirmed by α-tubulin. (B) Densitometry values of 3 independent experiments were normalised to total protein and expressed as percentage of control. Data are expressed as mean ± SEM. *p<0.05, *** p<0.001 *vs* control (Yazid *et al.*, 2013).

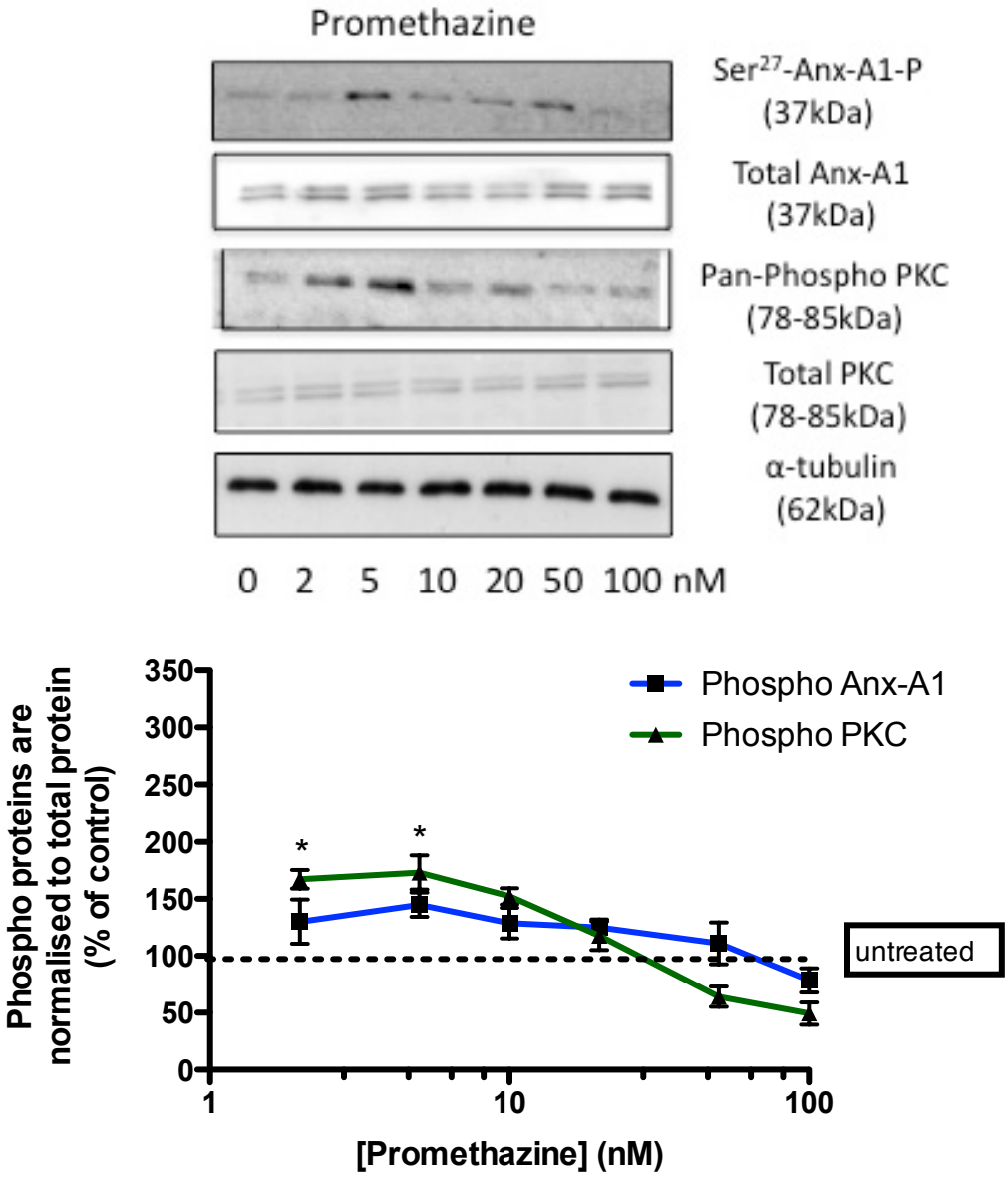


Figure 3.13: Promethazine only weakly promotes PKC-P at concentrations less than 10nM.

Whole CDMC lysate was prepared in the presence of 0 - 100nM of promethazine. Densitometry analysis shows that promethazine provokes similar trend of Anx-A1-P and PKC-P, whereby at concentrations from 20 - 100nM, the level of phosphorylation of both the proteins are almost abolished. Equal loading are confirmed by α-tubulin.

Densitometry values of 3 independent experiments were expressed as percentage of control. Data are expressed as mean ± SEM. Phospho PKC: * p<0.05 *vs* untreated.

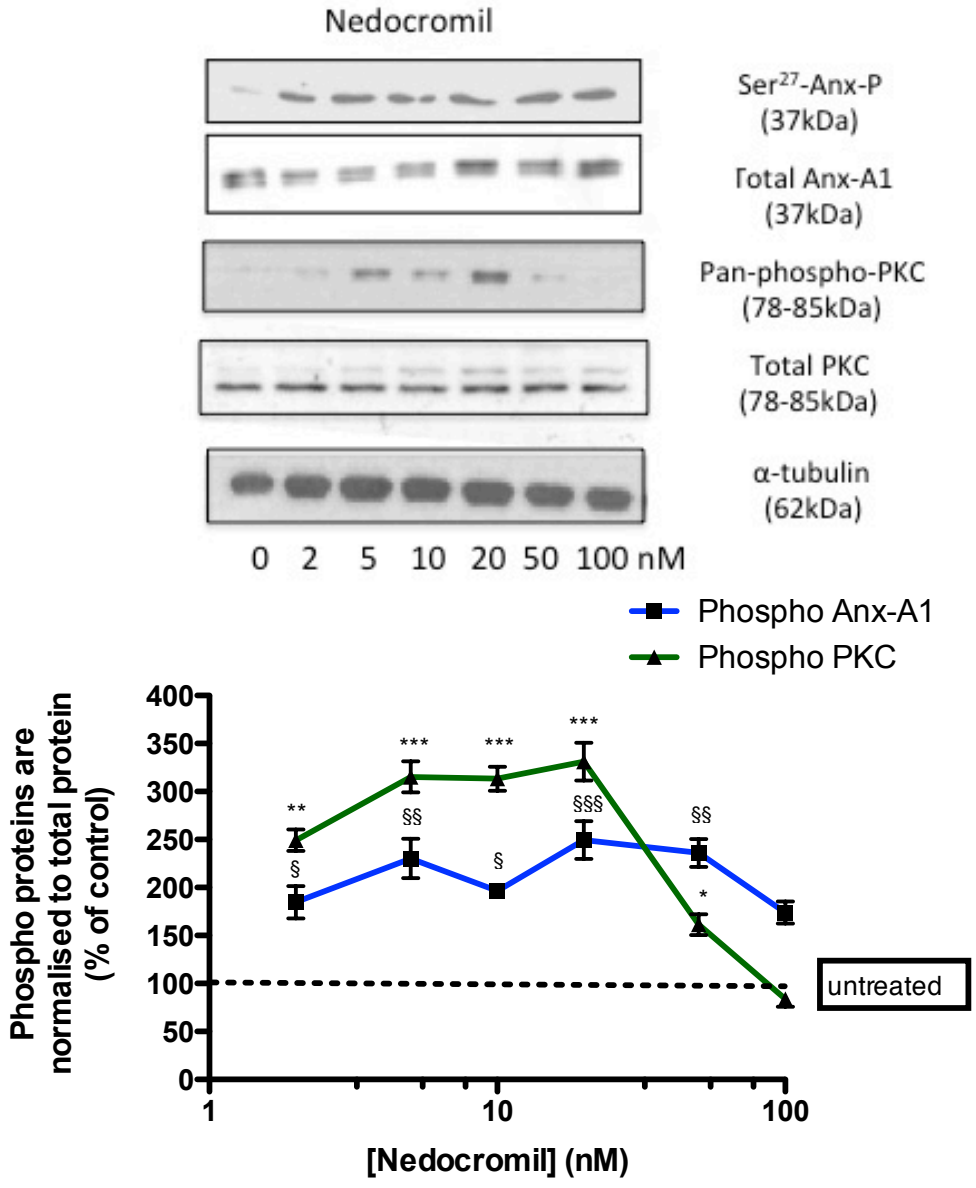


Figure 3.14: Nedocromil promotes PKC-P and Ser^{27}-Anx-A1-P at lower concentrations, however the level of Anx-A1 and PKC phosphorylation diminishes >20nM of nedocromil.

Detection of Anx-A1 and PKC phosphorylation was determined by Western blot. The phospho proteins were normalised to their respective total proteins. Densitometry values of 3 independent experiments were expressed as percentage of control. Data are expressed as mean ± SEM. Phospho PKC: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* untreated and phospho Anx-A1: § $p<0.05$, §§ $p<0.01$, §§§ $p<0.001$ *vs* untreated.

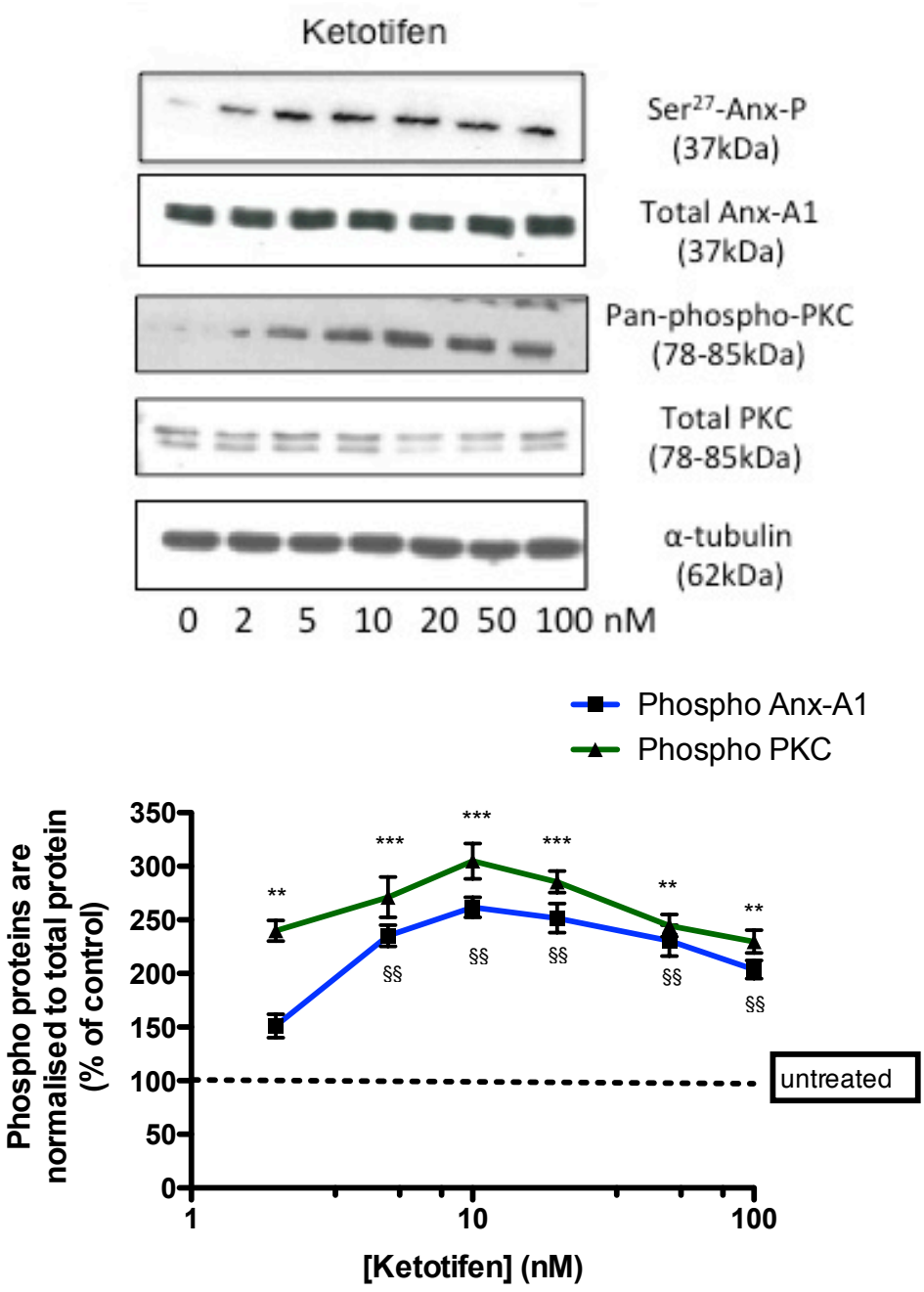


Figure 3.15: Ketotifen promotes Anx-A1 and PKC phosphorylation exhibiting a 'bell-shaped' concentration-response curve.

Densitometry values of 3 independent experiments were expressed as percentage of control. Data are expressed as mean ± SEM. Phospho PKC: ** $p<0.01$, *** $p<0.001$ *vs* untreated and phospho Anx-A1: §§ $p<0.01$ *vs* untreated.

3.2.3 PKC phosphorylation induced by dexamethasone (2nM) is prolonged by the combination with nedocromil (10nM) in U937 cells over time.

It has been hypothesised that the level of Anx-A1 phosphorylation and subsequently the amount that is exported out of the cell depends on the reciprocal interaction between PKC and PP2A enzyme. Therefore, drugs that prolong the expression of PKC would enhance the phosphorylation of Anx-A1. In line with this thought, the ability of nedocromil, dexamethasone or both drugs in combination, to promote phosphorylation of the PKC enzyme from 0-60 min time points in U937 cells was determined by Western blot. In Figure 3.16, nedocromil (10nM) promotes maximal PKC activity at 20 min (2.5 fold change from control) but the effect eventually wears off. Maximal phosphorylation of PKC upon treatment with dexamethasone (2nM) was observed at 10 min (2 fold-change) after which the effect fades with increasing time. Interestingly, when cells were treated with both nedocromil (10nM) and dexamethasone (2nM), there was a distinct increase of PKC phosphorylation, with maximal effect seen at 60 min (4 fold-change). This suggests that these drugs exhibit a strong synergistic action on PKC phosphorylation over time.

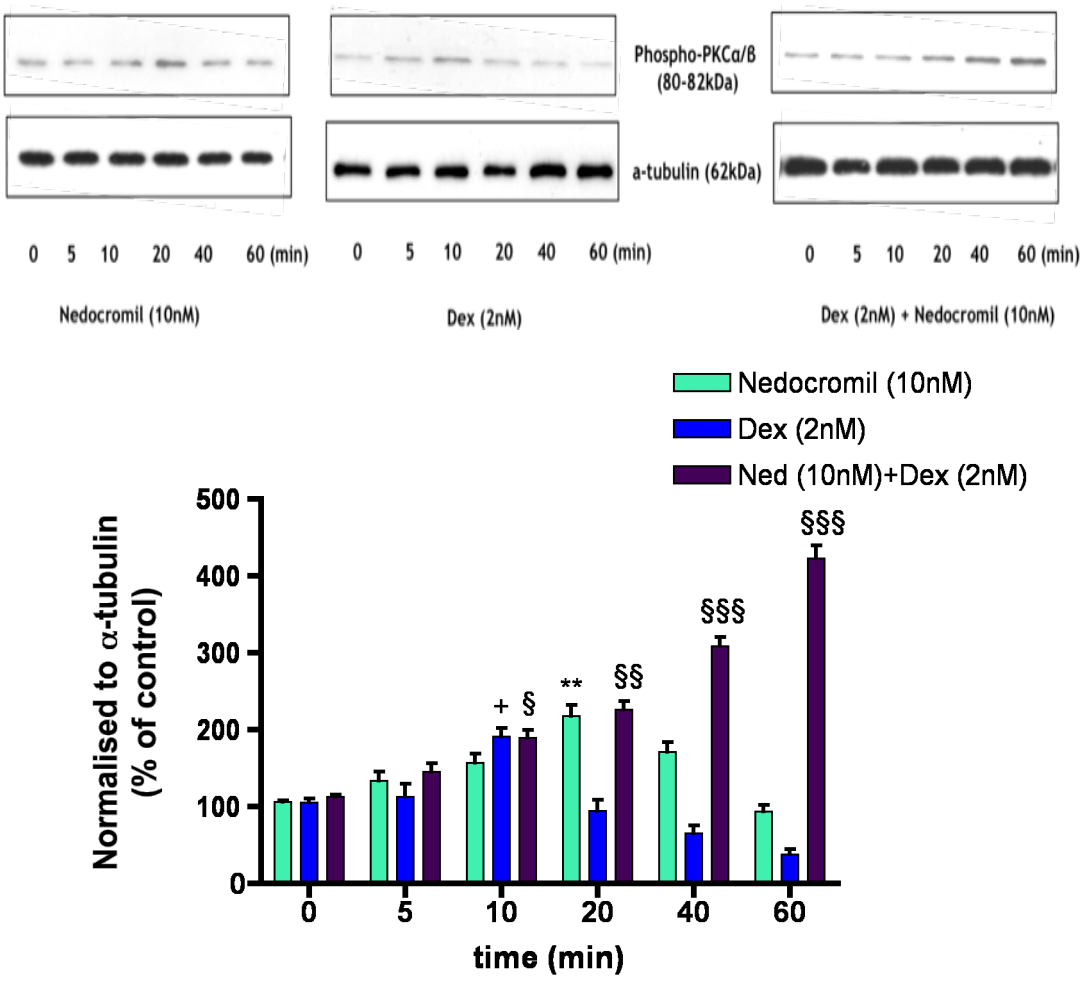


Figure 3.16: Nedocromil (10nM) in combination with dexamethasone (2nM) prolongs PKC phosphorylation in U937 cells.

Western blot analysis of PKC phosphorylation at different time points in U937 cells treated with nedocromil (10nM), dexamethasone (2nM) and both in combination. In the presence of dexamethasone and nedocromil, PKC phosphorylation was potentiated by 4 fold at 60 min. Representative blots of 3 independent experiments. (nedocromil: ** $p<0.01$ *vs* untreated, dexamethasone: $^{+}$ $p<0.05$ *vs* untreated and dexamethasone+nedocromil: § $p<0.05$, §§ $p<0.01$, §§§ $p<0.001$ *vs* untreated).

3.2.4 Phosphorylation of PKC is prolonged over time when CDMCs are treated with dexamethasone (2nM) in combination with nedocromil (10nM).

Since the synergistic effects of dexamethasone and nedocromil to prolong the PKC phosphorylation in the U937 cells were determined (Figure 3.16), the next question that arises was if similar effects could be reproduced in the CDMCs. The same protocol was repeated with CDMCs and Western blot was performed to analyse PKC phosphorylation across increasing time points. CDMCs were treated with either 10nM of nedocromil, 2nM of dexamethasone or both drugs in combination for various time points (0-60 min). Based on Figure 3.18 (panel A), it is evident that nedocromil was able to provoke maximal PKC phosphorylation at 10 min whereas treatment with dexamethasone produced an increase in the amount of PKC phosphorylation in the cell with no further effect seen beyond 5 min. However, both the drugs in combination were able to prolong the PKC phosphorylation (4 fold) across 1h time point. The results obtained from CDMCs were similar to that performed on U937 cells, suggesting that dexamethasone and nedocromil synergistically promote PKC phosphorylation across increasing time and this mechanism is independent of the cell type.

The increments in the phosphorylated isoform of PKCα/β would be indicative of the protein mobilization and secretion at the plasma membrane when activated and Anx-A1 is likewise recruited to the membrane upon

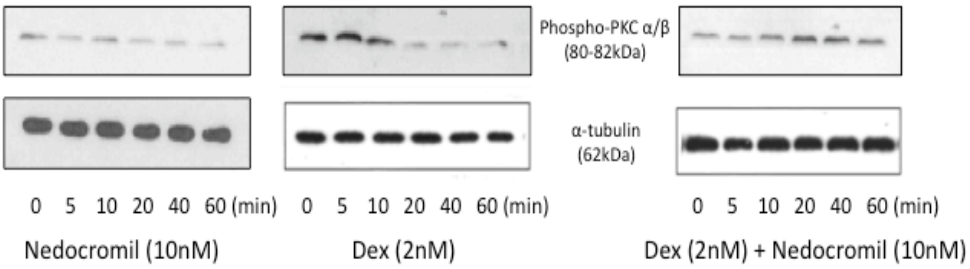
phosphorylation at Ser^{27} residue. To investigate the effect of dexamethasone alone or in combination of nedocromil on the patterns of intracellular trafficking of PKC and Anx-A1 across increasing time points at a fine level of detail, CDMCs were utilised and the effects of the drug treatment were analysed by confocal microscopy.

Figure 3.18 (panel A, B and C) shows an experiment where CDMCs were stained for total PKC (red; Alexa Fluor 546; anti-rabbit) and total Anx-A1 (green; Alexa Fluor 488; anti-mouse). CDMCs were treated with dexamethasone (2nM) alone or in combination with nedocromil (10nM) across various time points. The untreated cells (panel A), show similar cytoplasmic distribution of total PKC and Anx-A1 across 5, 10 and 20 min. PKC and Anx-A1 was observed mainly in the cytoplasmic or peri-nuclear region apparently associated with vesicles. Both these proteins were not observed at the plasma membrane since there were no co-localisation between PKC and Anx-A1.

Figure 3.18; B shows that when CDMCs were treated with dexamethasone for 5 min, there is a certain degree of co-localisation at membrane level between total PKC and Anx-A1. However, this effect was short-lived, fading after 10 min. Interestingly, exposure of cells to nedocromil in the presence of dexamethasone (Figure 3.18; C) for 5 min shows substantial amounts of PKC has translocated to the plasma membrane and is co-localised with Anx-A1 and this effect is prolonged across increasing time point, suggesting that nedocromil was able to prolong the distribution of total

PKC at membrane level. Since a combination of nedocromil and dexamethasone potentiates Anx-A1 phosphorylation and secretion, apparently through an increase of PKCα/β activation at the membrane, it could be conjectured that this drug might act as a phosphatase (PP2A) inhibitor that prevents PKCα/β dephosphorylation at the membrane in CDMCs. It has been shown in the U937 cells that the catalytic activity of PKCα/β is limited by membrane phosphatases, in particular PP2A (Yazid *et al.*, 2009). The confocal images are in agreement with the Western blots whereby nedocromil in combination with dexamethasone was able to prolong the 'dwell' time of PKC phosphorylation in CDMCs.

A)



B)

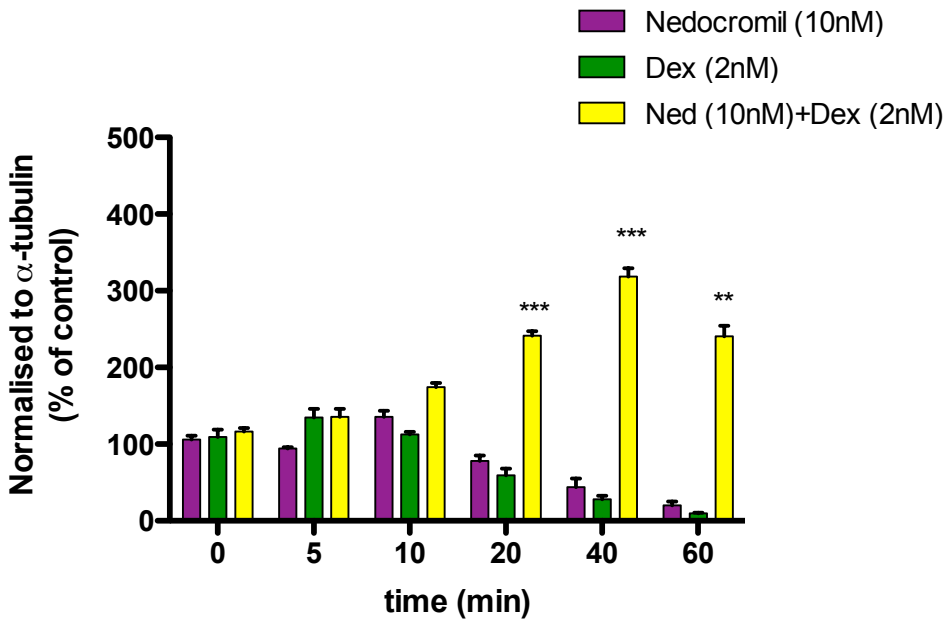
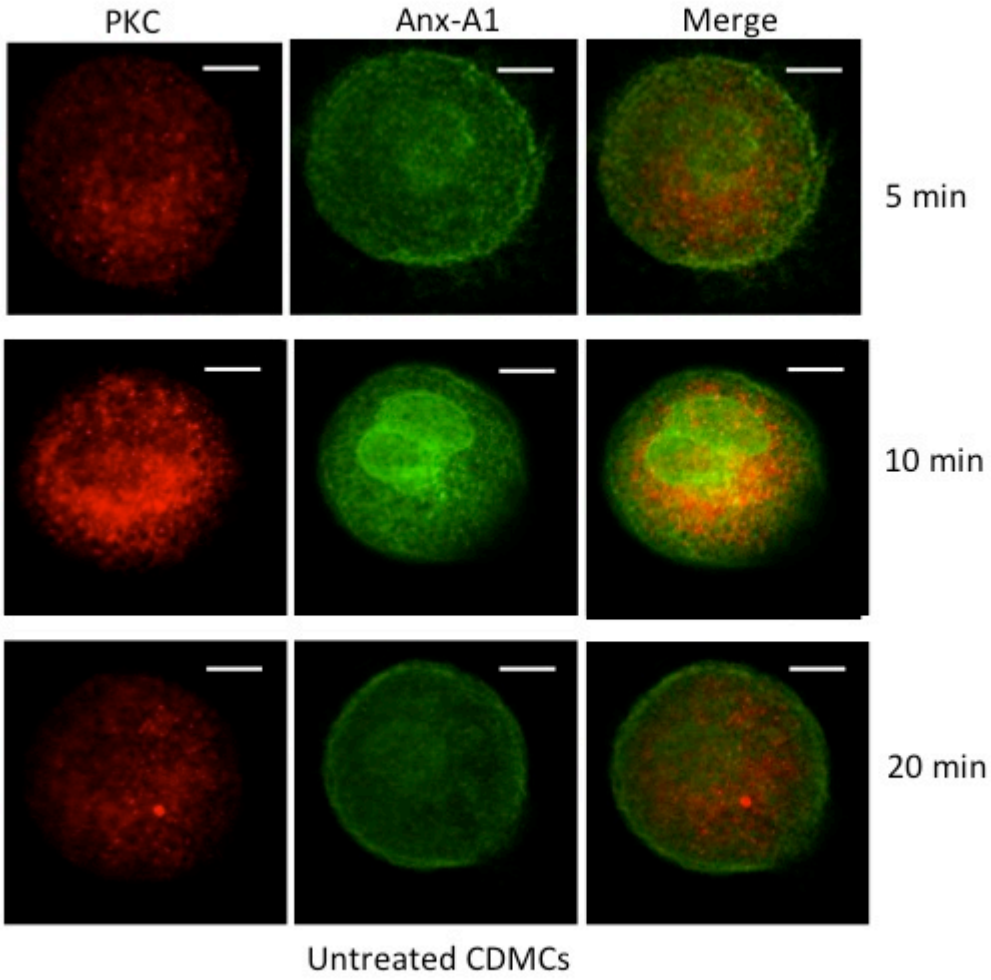


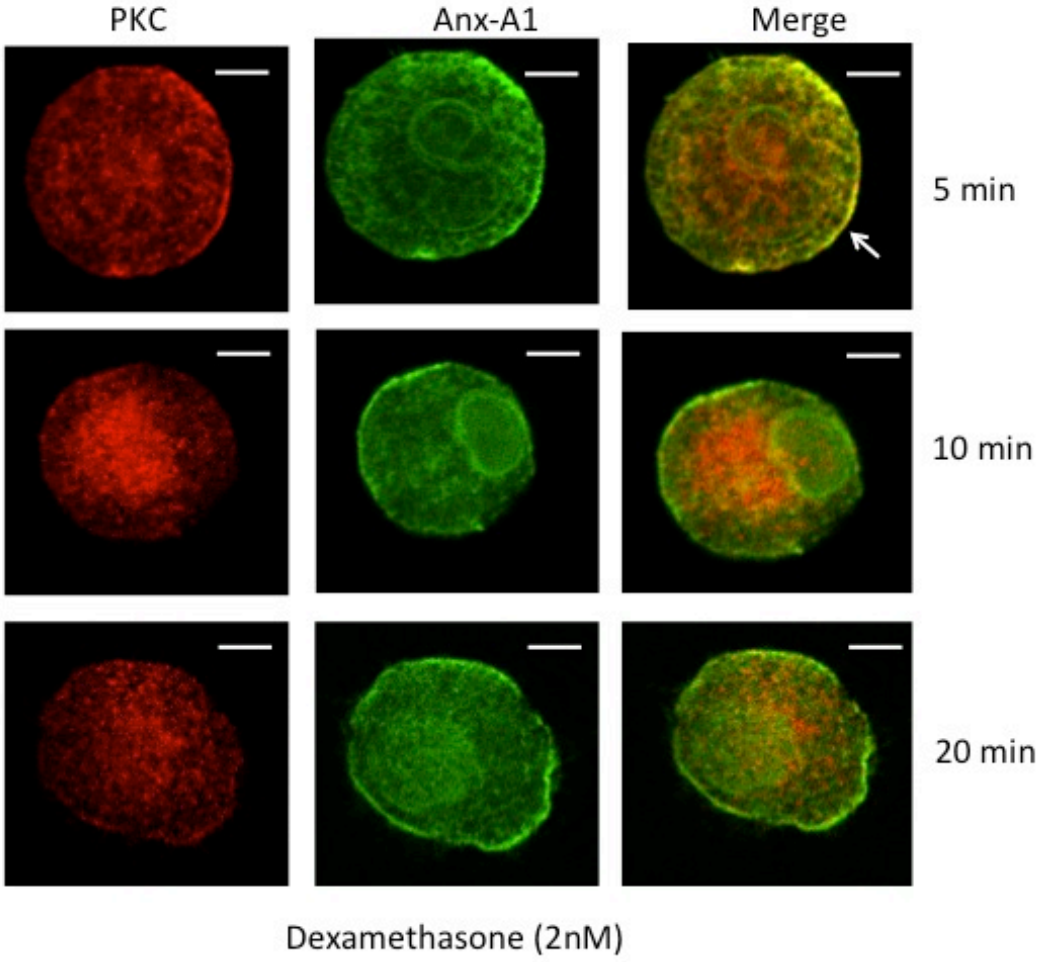
Figure 3.17: Nedocromil (10nM) in combination with dexamethasone (2nM) prolongs PKC phosphorylation in CBMCs.

CBMCs whole lysate were treated with nedocromil (10nM), dexamethasone (2nM) or both in combination and processed for Western blot analysis of PKC phosphorylation at different time points. In the presence of dexamethasone and nedocromil, PKC phosphorylation was potentiated by 4 fold at 40 min. Densitometry values of 3 independent experiments were expressed as percentage of control (dexamethasone+nedocromil: ** $p<0.01$, *** $p<0.001$ *vs* untreated).

A)



B)



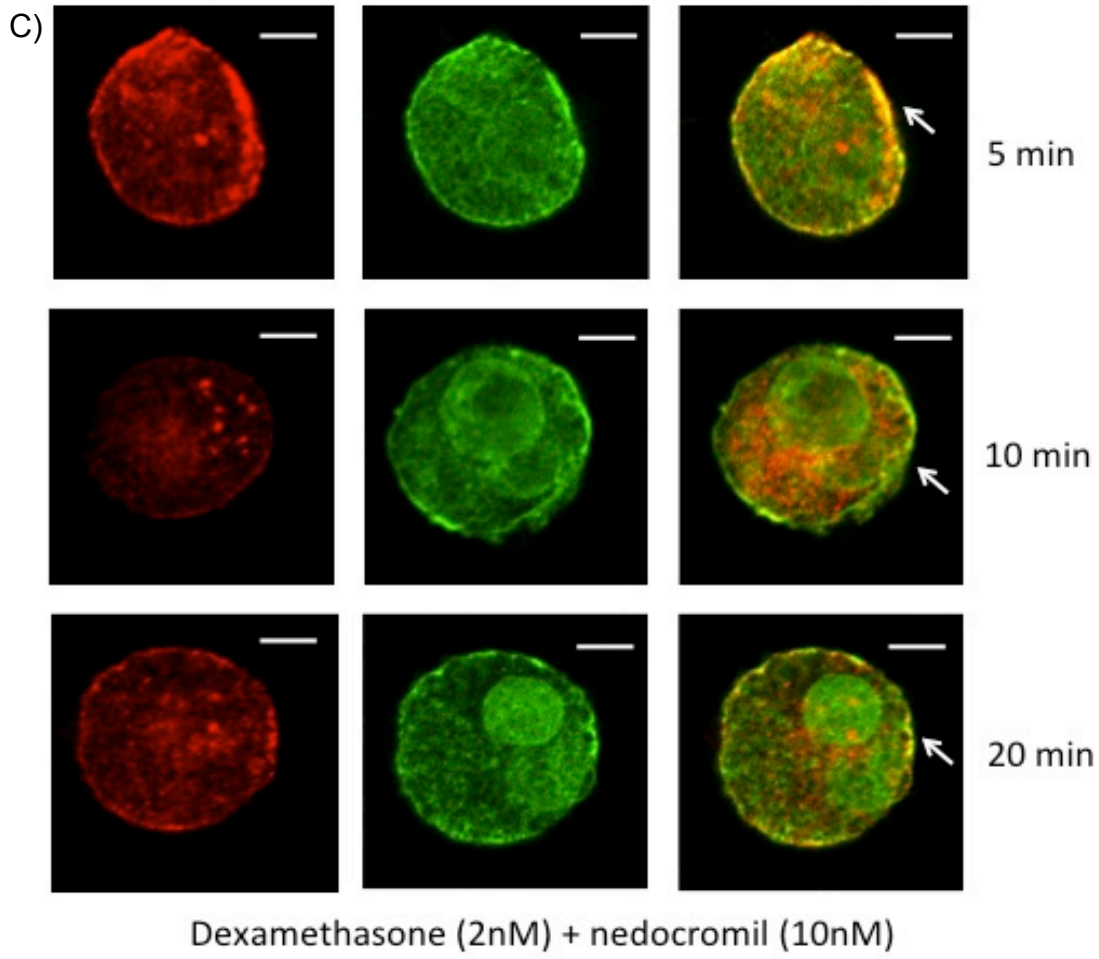


Figure 3.18: PKC 'dwell' time at the cell membrane increases when nedocromil is combined with dexamethasone.

The CDMCs were plated at a density of $2x10^5$ cells and prepared following the protocol described in the methodology sections. Cells were stained for total PKC (red; Alexa Fluor 546; anti-rabbit) and total Anx-A1 1B (green; Alexa Fluor 488; anti-mouse). The three columns represent the information from the two colour channels and the merged channel. The CDMCs were either treated with dexamethasone (2nM) for 5, 10 and 20 min or treated with both nedocromil (10nM) and dexamethasone (2nM) for the same time points. The white arrows indicate co-localisation between PKC and Anx-A1. The images were taken at 63x oil magnification. The scale bars indicate 10μm. This figure is representative of three independent experiments.

3.3 COMPOUND 48/80 AS A MAST CELL SECRETAGOGUE.

3.3.1 Compound 48/80-induced degranulation was prevented when CDMCs were pre-treated with nedocromil (10nM).

Compound 48/80 is a N-methyl-p-methoxyphenethylamine condensation producer that is commonly used to degranulate mast cells. Prior to using this compound for our in-vitro assays, the optimal concentration of compound 48/80 that induces CDMCs degranulation was first established. In order to do this, the cells were treated with a range of compound 48/80 concentrations (5 - 20 µg/ml) and the morphology of the cells was observed under phase contrast microscope. The CDMCs degranulation was observed and counted in a blinded manner. Figure 3.19 shows distinct characteristics of mast cell degranulation upon stimulation with varying concentrations of compound 48/80 (upper panel). Inverted light microscope images of untreated CDMC, were observed to be oval in shape with distinct cell membrane and cytoplasm containing fine granules (A). Upon stimulation with compound 48/80 at 5µg/ml, cells exhibit marked membrane protrusion (black arrow), which indicates the beginning of degranulation (B). CDMCs were observed to have extruded granules (black arrow) near the cell surface when stimulated with 10µg/ml of compound 48/80 (C). CDMCs were seen to markedly degranulate (black arrow) when treated with 20µg/ml of Compound 48/80 (D). Interestingly, CDMCs pre-treated with mast cell stabiliser, nedocromil (10nM) for 5 min prior to compound 48/80 (20µg/ml) stimulation

did not degranulate (E). Figure 3.19 (lower panel) shows that 10μg/ml of compound 48/80 induces approximately 45% of CDMCs degranulation. Since 10μg/ml of Compound 48/80 produces half maximal effect of mast cell degranulation, this concentration was used for the subsequent experiments.

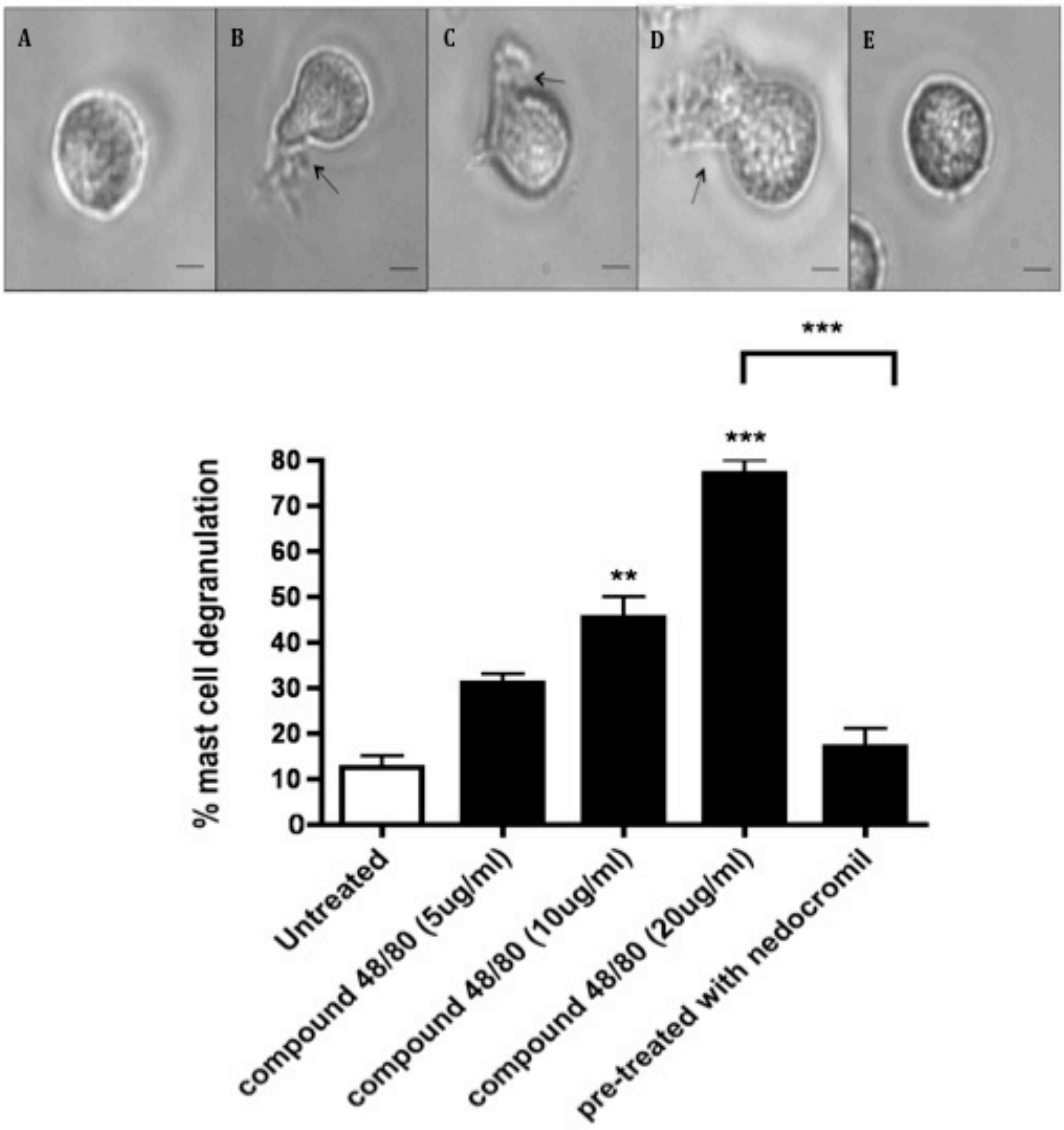


Figure 3.19: Pre-treatment of nedocromil (10nM) for 5 min prevented compound 48/80-induced CDMC degranulation.

Upper panel: Inverted light microscope image of CDMCs stimulated with compound 48/80. A) Unstimulated CDMCs, B) CDMCs treated with 5µg/ml of compound 48/80, C) CDMCs stimulated with 10µg/ml compound 48/80, D) CDMCs stimulated with 20µg/ml of compound 48/80, E) CDMCs pre-treated with 10nM of nedocromil for 5 min and stimulated with 20µg/ml of compound 48/80 for 10 min. Lower panel: The bar graph depicts the percentage of human mast cell degranulation upon stimulation with compound 48/80. Nedocromil significantly ($p<0.001$) inhibits compound 48/80-

induced degranulation in CDMCs. (**p<0.01 and ***p<0.001). Data are expressed as mean ± SEM of n=3 independent experiments. Bars indicate 10μm.

3.3.2 Compound 48/80 promotes histamine and phospho-Anx-A1 release in a concentration-dependent manner in CDMCs.

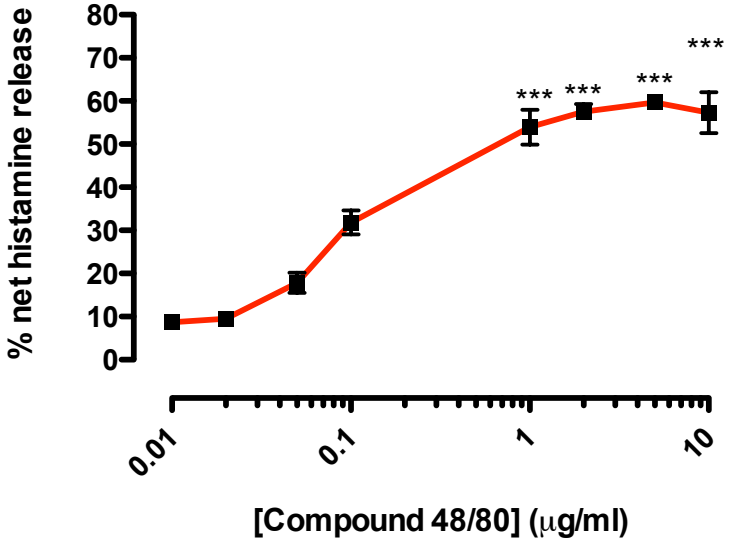
Since PKC itself is activated during mast cell degranulation, the next question that arises was whether the stimulation of mast cells by 48/80 would be a sufficient stimulus to promote phosphorylation and release of Anx-A1. The previous experiment showed that compound 48/80 elicits mast cell degranulation. Thus, the ability of compound 48/80 to release histamine in CDMCs at varying concentrations was assessed. Since, compound 48/80 is able to promote approximately 80% of mast cell degranulation at 20μg/ml (Figure 3.19), the stimulation of histamine release at lower concentrations of compound 48/80 ranging from 0.01 - 10 μg/ml was investigated.

Figure 3.20 (panel A) indicates that compound 48/80 produces a linear increase in histamine release in concentrations ranging from 0.01 to 0.1 μg/ml, but thereafter, from concentrations 1 - 10 μg/ml, the secretagogue reaches a plateau at about 50% of net histamine release

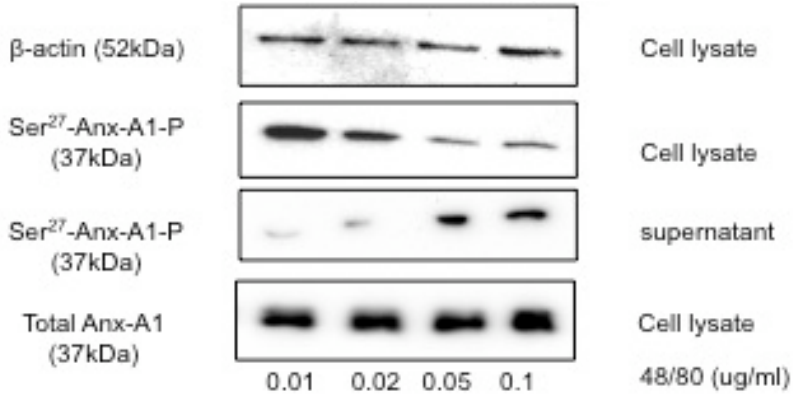
As lower concentrations of compound 48/80 (0.01 to 0.1 μg/ml) promotes only about 10 - 30% of net histamine release, these were deemed ideal to investigate the ability of compound 48/80 to induce Anx-A1 phosphorylation as it was essential to have a working concentration of compound 48/80 that did not induce maximal degranulation. The Western blot in Figure 3.20 (panel B) shows that compound 48/80 induces a concentration-

dependent decrease in Anx-A1 phosphorylation at Ser^{27} residue, and this is reflected in the amount of phosphorylated Anx-A1 that is then released out into the medium.

A)


% net histamine release
80
70
60
50
40
30
20
10
0
***
***
***
***
0.01
0.1
1
10
[Compound 48/80] (μg/ml)

B)


β-actin (52kDa)
Cell lysate
Ser27-Anx-A1-P (37kDa)
Cell lysate
Ser27-Anx-A1-P (37kDa)
supernatant
Total Anx-A1 (37kDa)
Cell lysate
0.01
0.02
0.05
0.1
48/80 (ug/ml)

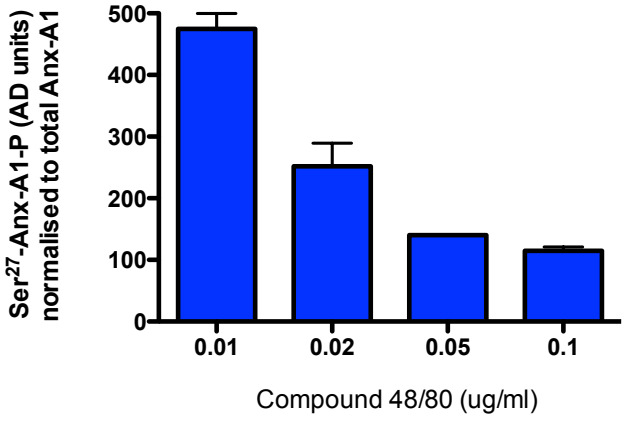

Ser27-Anx-A1-P (AD units) normalised to total Anx-A1
500
400
300
200
100
0
0.01
0.02
0.05
0.1
Compound 48/80 (ug/ml)

Figure 3.20: Compound 48/80 promotes the release of histamine and exerts the phosphorylation and release of Anx-A1 in a concentration-dependent manner in CDMCs.

A) Lower concentrations of compound 48/80 (0.01-0.1µg/ml) induces 10 - 20% of histamine release, whilst approximately 30% of histamine release was induced by 1µg/ml of compound 48/80 and it reaches a plateau thereafter at ~50% of net histamine release. Data are expressed as mean ± SEM of n=3 independent experiments, (***$p<0.001$). B) Phosphorylation and release of Anx-A1 is stimulated in a concentration-dependent manner by compound 48/80. Exposure to compound 48/80 (0.01 - 0.1µg/ml) caused a rapid concentration-dependent degranulation and a concomitant increase in phosphorylation of Anx-A1 at Ser^{27} and a release of the protein into the surrounding medium. Densitometry values of 3 independent experiments are expressed as mean ± SEM.

3.3.3 Characterisation of compound 48/80 on Anx-A1 phosphorylation across time.

To further provide additional support for the notion that nedocromil 'stabilises' mast cells by inducing Anx-A1 phosphorylation in stimulated cells, Western blots were performed on CDMCs lysates that were either stimulated with compound 48/80 (0.05µg/ml) alone, treated with nedocromil (10nM) alone or pre-treated with nedocromil (10nM) prior to compound 48/80 stimulation across increasing time points.

Figure 3.21 shows that compound 48/80 induces a maximal Anx-A1 phosphorylation (1.4 fold change from control) at 5 min which thereafter decays with time indicating that the cells would have degranulated. CDMCs treated with nedocromil (10nM) alone shows maximal Anx-A1

phosphorylation (2.7 fold change from control) at 5 min and this subsequently decays around the next 40 min. However, pre-treatment of nedocromil prior to compound 48/80 stimulation was able to augment Anx-A1 phosphorylation at all time points measured with the maximal effect (4.5 fold change from control) observed at 40 min. This result indicates that nedocromil is able to prolong Anx-A1 phosphorylation in CDMCs stimulated by compound 48/80.

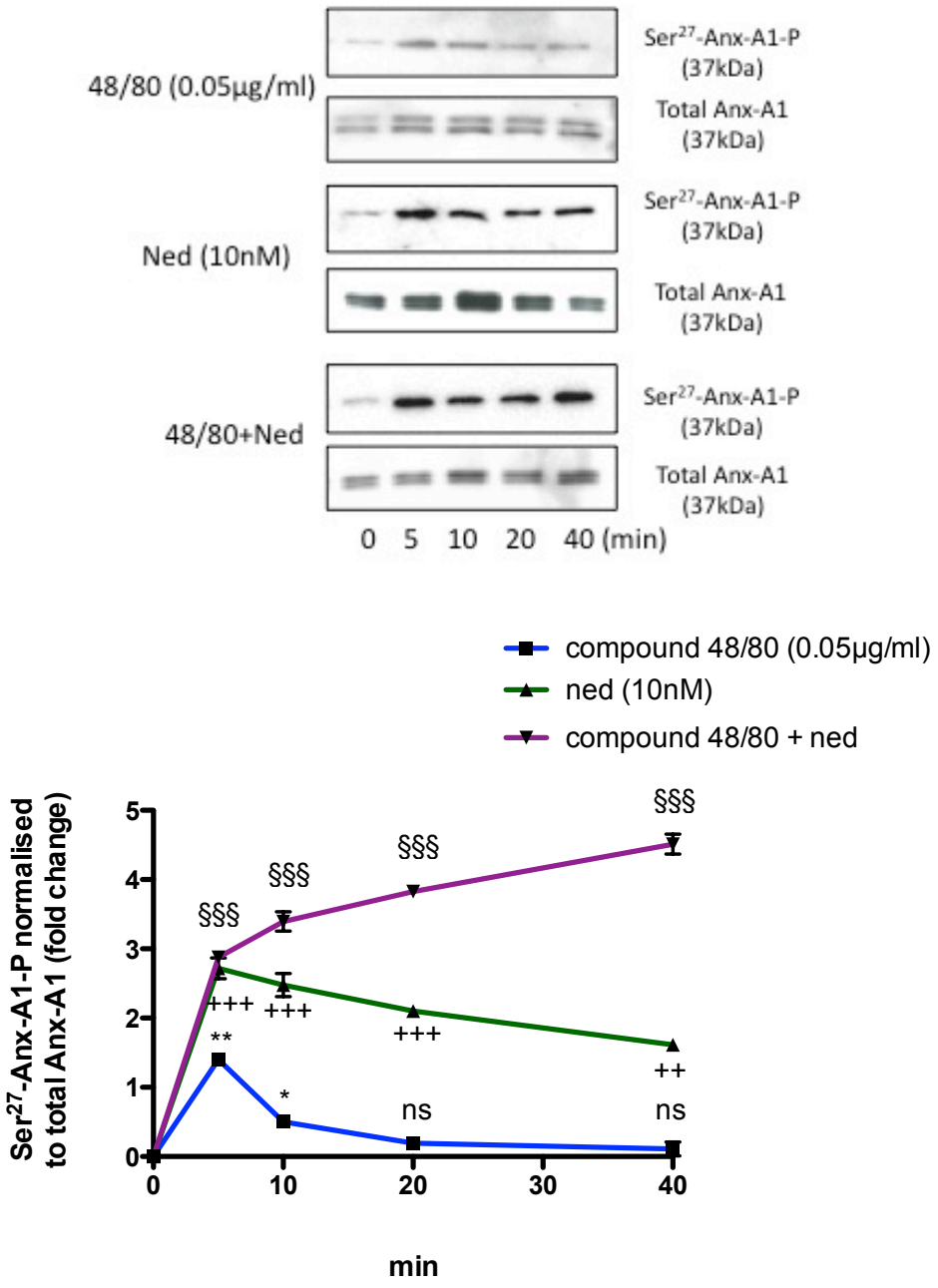


Figure 3.21: Nedocromil potentiates Ser^{27}-Anx-A1 phosphorylation with time in stimulated CDMCs.

Compound 48/80 (0.05µg/ml) increases Anx-A1 phosphorylation with a maximal effect seen at 5 min and pre-treatment with nedocromil alone potentiates Anx-A1 phosphorylation at 5 min. However nedocromil (10nM) prolonged the effect of compound 48/80 by increasing Anx-A1 phosphorylation in a time-dependent manner.

Data are expressed as mean ± SEM. (n=3 independent experiments, * $p<0.05$, ** $p<0.01$; ++ $p<0.01$, +++ $p<0.001$; §§§ $p<0.001$ *vs* control).

3.4 MECHANISM OF ACTION OF NEDOCROMIL IN CDMCS.

3.4.1 Nedocromil (10nM) inhibits β-hexosaminidase, histamine and PGD_2 release by CDMCs but the effect is reversed by neutralising anti-Anx-A1 monoclonal antibody.

To investigate further the mechanism by which nedocromil suppress mediator release from CDMCs, biochemical and immuno-neutralising techniques was used.

Firstly, it was determined whether the cultured CDMCs responded with the release of β-hexosaminidase, which is commonly used as an indicator of degranulation in mast cells. To determine the role of Anx-A1 in maintaining the stability of mast cells, the effect of a specific neutralising monoclonal anti-Anx-A1 antibody was tested. CDMCs were pre-incubated for 20 min with 20μg/ml neutralising anti-Anx-A1, or an irrelevant isotype-matched, monoclonal antibody. Figure 3.22 (panel A) shows that unstimulated CDMCs spontaneously released 8.9 ± 0.3% of β-hexosaminidase. However, in the presence of neutralising anti-Anx-A1 monoclonal antibody, the CDMCs released 43.6 ± 3.6% of β-hexosaminidase, suggesting that without extracellular Anx-A1, CDMCs undergoes spontaneous degranulation. The irrelevant isotype control was inactive in this system.

Next, the effect of the co-incubation of stimulated CDMCs with neutralising anti-Anx-A1 (or irrelevant) monoclonal antibody on the effect of the inhibitory action of 10nM nedocromil on β-hexosaminidase release was investigated. Figure 3.22;B shows that nedocromil significantly ($p<0.01$) inhibits the β-hexosaminidase release by 32.3% relative to stimulated cells. However this inhibitory effect of nedocromil was completely abolished in the presence of Anx-A1 neutralising Ab, suggesting that the acute inhibition of β-hexosaminidase release from CDMCs by nedocromil is Anx-A1 dependent. The irrelevant isotype control was inactive in this system.

The above experiment demonstrated that the acute effects of nedocromil as an inhibitor of mast cell degranulation in human CDMCs appeared to be mediated by Anx-A1. Histamine and PGD_2 assays were carried out to determine the mechanism by which nedocromil suppressed these mediators. The results on histamine release are presented as the percent inhibition in order to normalise the baseline differences between cultures that might have occurred due to variation among the individual batches of CDMCs obtained from different donors (Yamaguchi *et al.*, 1999; Tachimoto *et al.*, 2000).

Based on the assay, the spontaneous release of histamine from unstimulated CDMCs was approximately 3.5 ± 6.1% and this readout was taken into consideration when the net histamine level was calculated (see materials and methodology section). It is noteworthy to mention that, PGD_2 was not released in unstimulated CDMCs as this mediator release is only

triggered in the presence of stimulus. Figure 3.23 shows that 10 min stimulation by 10µg/ml compound 48/80 in CDMCs provoked a release of approximately 40% of net histamine release and 1331 ± 237.8 pg ml^{-1} of PGD_2. Pre-treatment of cells with nedocromil prior to compound 48/80 stimulation, was able to significantly inhibit the release of histamine ($p<0.001$; 10.7 ± .08 %) and PGD_2 ($p<0.01$; 657.7 ± 38.2 pg ml^{-1}) in CDMCs. However, in the presence of neutralising anti-Anx-A1 monoclonal antibody, the inhibitory action of nedocromil was reversed. Neither the neutralising anti-Anx-A1 monoclonal antibody nor the irrelevant isotype control alone had any significant effect on the release of mediators. Since the neutralising anti-Anx-A1 monoclonal antibody has effects on its own on the mast cell degranulation, one may argue that the effects of nedocromil is due to the presence of neutralising anti-Anx-A1 monoclonal antibody. It could be possible that the neutralising anti-Anx-A1 monoclonal antibody is masking the effects of nedocromil in these cells. However, previous study had demonstrated that nedocromil was not effective in the Anx-A1 KO mast cells (Yazid *et al.*, 2013), thus further reiterating that the inhibitory effects of nedocromil is dependent on the presence of Anx-A1.

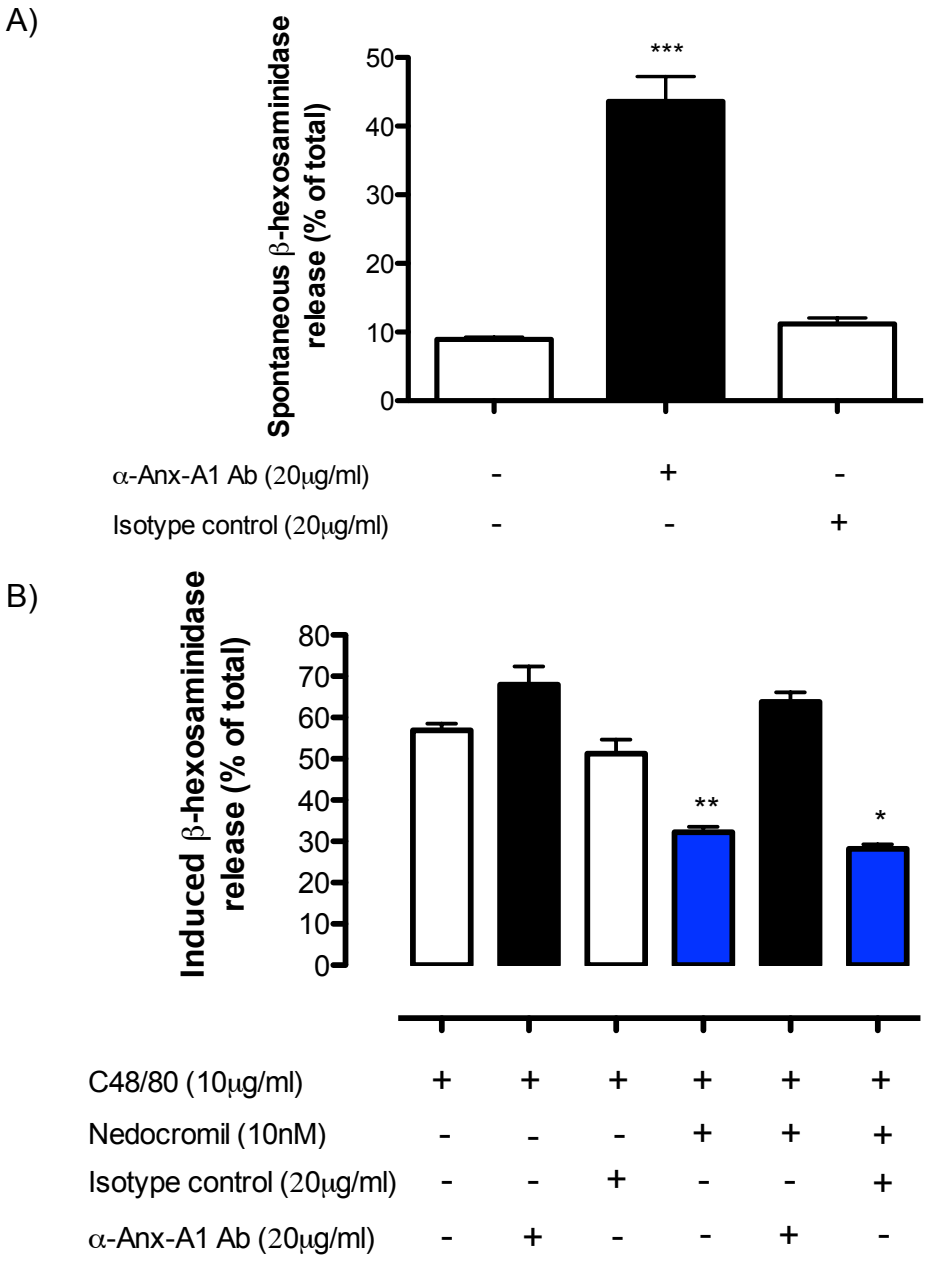


Figure 3.22: CDMC degranulation was assessed by measuring β-hexosaminidase release.

A) The supernatants from CDMCs were assessed for β-hexosaminidase assay and based on the assay, these unstimulated cells release about 43.6% of β-hexosaminidase in the presence of neutralising anti-Anx-A1 mAb. B) Nedocromil significantly ($p<0.01$) inhibits the β-hexosaminidase release by 32.3% relative to stimulated cells. However this effect was completely abolished in the presence of

neutralising anti-Anx-A1 mAb. Data were analysed using one-way analysis of variance (ANOVA), followed by a Bonferonni post-hoc test, ** p<0.01, *** p<0.001 *vs* unstimulated. Data are expressed as mean ± SEM of n=3 independent experiments.

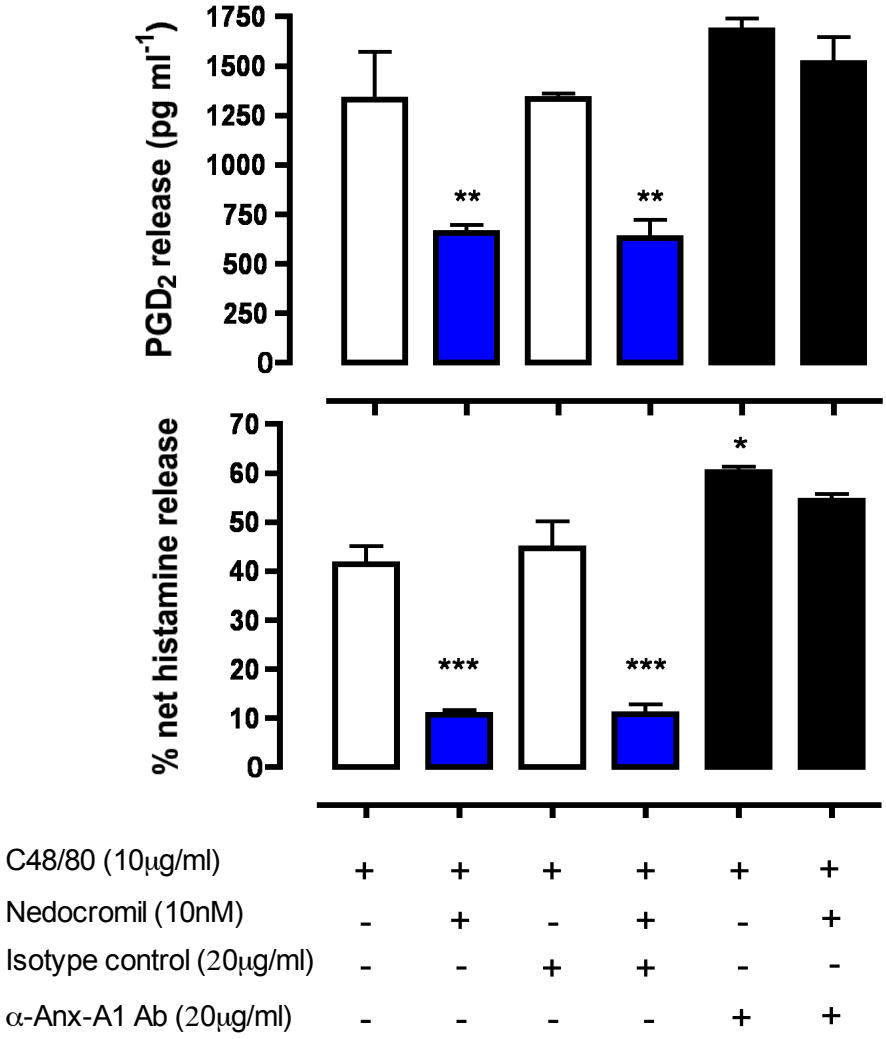


Figure 3.23: Nedocromil inhibits histamine and PGD_2 release but is not effective in the presence of neutralising anti-Anx-A1 mAb.

CDMCs were plated at a density of $2x10^5$ cells per well and the stipulated groups were administered with neutralising anti-Anx-A1 mAb or an irrelevant isotype control. Subsequently, the cells were pre-treated with nedocromil (10nM) for 5 min prior to compound 48/80 (10µg/ml) stimulation for 10 min. Supernatants were collected from

the samples and assessed for histamine and PGD_2 assay by ELISA. (* signifies $p<0.05$, ** signifies $p<0.01$, *** signifies $p<0.001$ relative to control). Data are expressed as mean ± SEM of n=3 independent experiments.

3.4.2 Inhibition of histamine and PGD_2 in murine BMDMCs by nedocromil is blocked by neutralising anti-Anx-A1 monoclonal antibody.

It has been shown in the previous experiments in CDMCs that the acute effects of nedocromil were Anx-A1 mediated. To eliminate the possibility that the role of Anx-A1 in the inhibitory actions of nedocromil was a feature unique to CDMCs, BMDMCs isolated from transgenic mice in which the Anx-A1 gene was globally deleted were used.

Murine mast cells from wild type and Anx-A1 null mice were cultured and matured from bone marrow precursors as described. Figure 3.24 shows the electron micrographs of the WT and Anx-$A1^{-/-}$ BMDMCs that were used in this study (courtesy of Dr Yazid, UCL). The BMDMCs from WT mice was first utilised to establish whether these cells behaved in the same manner to the human CDMCs. The spontaneous release of histamine in the unstimulated WT BMDMCs was approximately 5.9 ± 2.3 % of the total histamine content, whilst 8.1 ± 3.4% was released in the KO BMDMCs. Figure 3.25 A and B shows that nedocromil (10nM) significantly ($p<0.001$) inhibits histamine and PGD_2, (approximately 40% and 30% from control respectively) to a similar extent to that observed in the CDMCs. Once again, in the presence of

neutralising anti-Anx-A1 monoclonal antibody, nedocromil was inactive in this model, suggesting that nedocromil is dependent on Anx-A1 to exert its anti-inflammatory pathway

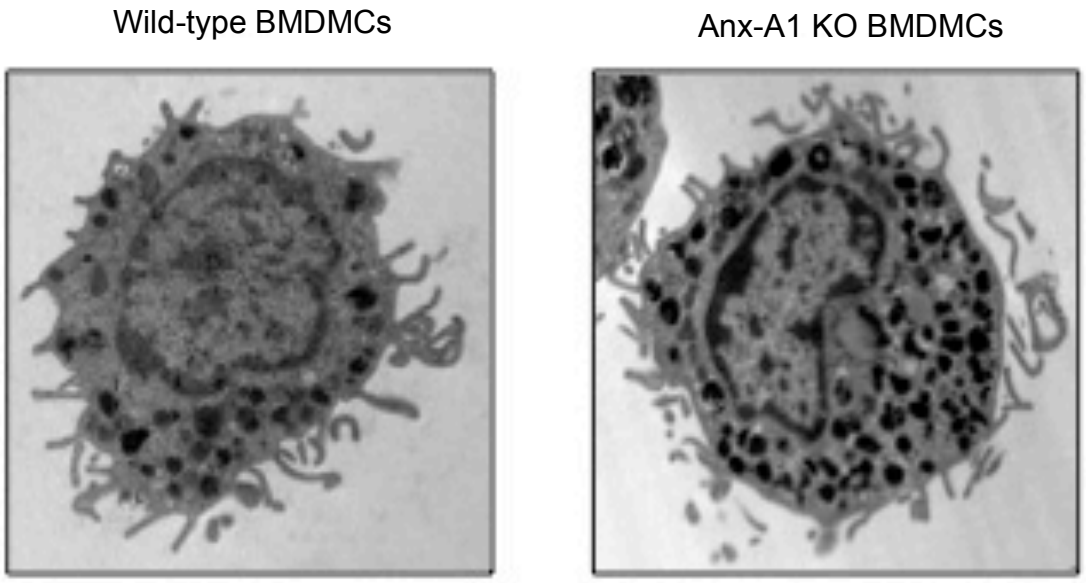


Figure 3.24: Electron micrograph of BMDMCs isolated from the wild type and Anx-A1 null mice.

The electron micrographs show that the BMDMCs have typical mast cell morphology. The cytoplasm is dense with secretory granules and the nucleus is centrally located whilst the cell surface is covered with fine microvilli. There was no obvious difference between the phenotype but planimetric analysis revealed some differences in granule morphology.

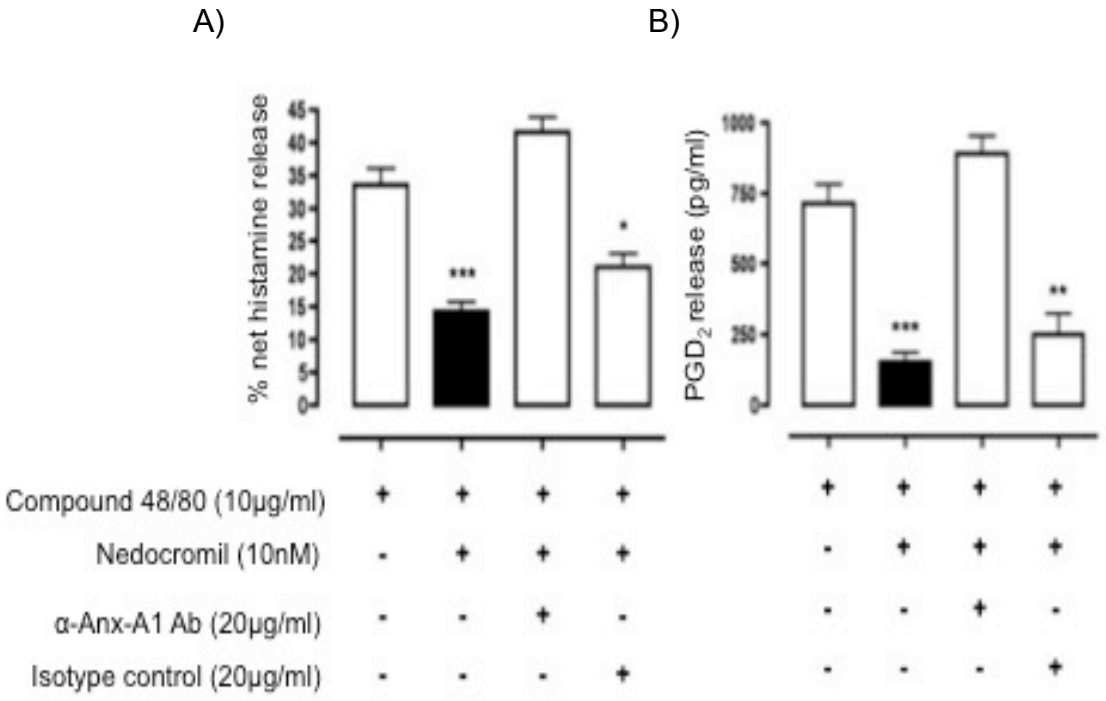


Figure 3.25: The inhibitory effect of nedocromil of compound 48/80 stimulated histamine and PGD_2 release from BMDMCs is Anx-A1 dependent

BMDMCs from the WT strain of BALB/C mice were prepared and stimulated with compound 48/80 (10µg/ml). Nedocromil (10nM) was administered alone or in the presence of either Anx-A1 neutralising antibody or an irrelevant isotype control (20µg/ml). Histamine (A) and PGD_2 (B) were assessed by ELISA assay. Nedocromil significantly inhibits the release of histamine and PGD_2 but in the presence of Anx-A1 neutralising antibody, this effect was reversed. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* control). Data are expressed as mean ± SEM of n=3 independent experiments. (Yazid *et al.*, 2013).

3.4.3 Hr-Anx-A1 inhibits histamine and PGD_2 release in $Anx^{-/-}$ BMDMCs.

The inhibitory effects of a range of 5 concentrations of nedocromil (0.5 - 10nM) using two different protocols in which mediator release was stimulated with either DNP-IgE/anti DNP induced IgE cross-linking or by compound 48/80 were tested. Figure 3.26; panel A and B shows that nedocromil produced a concentration-dependent inhibition of histamine and PGD_2 release, respectively, from WT BMDMCs when this was elicited by either anti DNP-IgE/DNP-BSA cross-linking in the case of pre-sensitised cells, or compound 48/80 in un-sensitised cells. However, nedocromil was inactive in the KO BMDMCs, which further points to a crucial role of Anx-A1 in the acute inhibitory actions of nedocromil.

To confirm that this was not due to the fact that the cells cultured from the Anx-A1 null mice were not responsive to Anx-A1, the cells were treated with a range of hr-Anx-A1 concentrations (0.1 - 20nM). Figure 3.26; panel C shows that the exogenous Anx-A1 produces a concentration-dependent inhibition of both histamine and PGD_2 in the KO BMDMCs. This data shows that whilst the BMDMCs from the Anx-A1 null mice was not able to release Anx-A1, these cells retain their sensitivity to the exogenous hr-Anx-A1 protein, which inhibits histamine and PGD_2 release with an IC_{50} value of approximately 5nM.

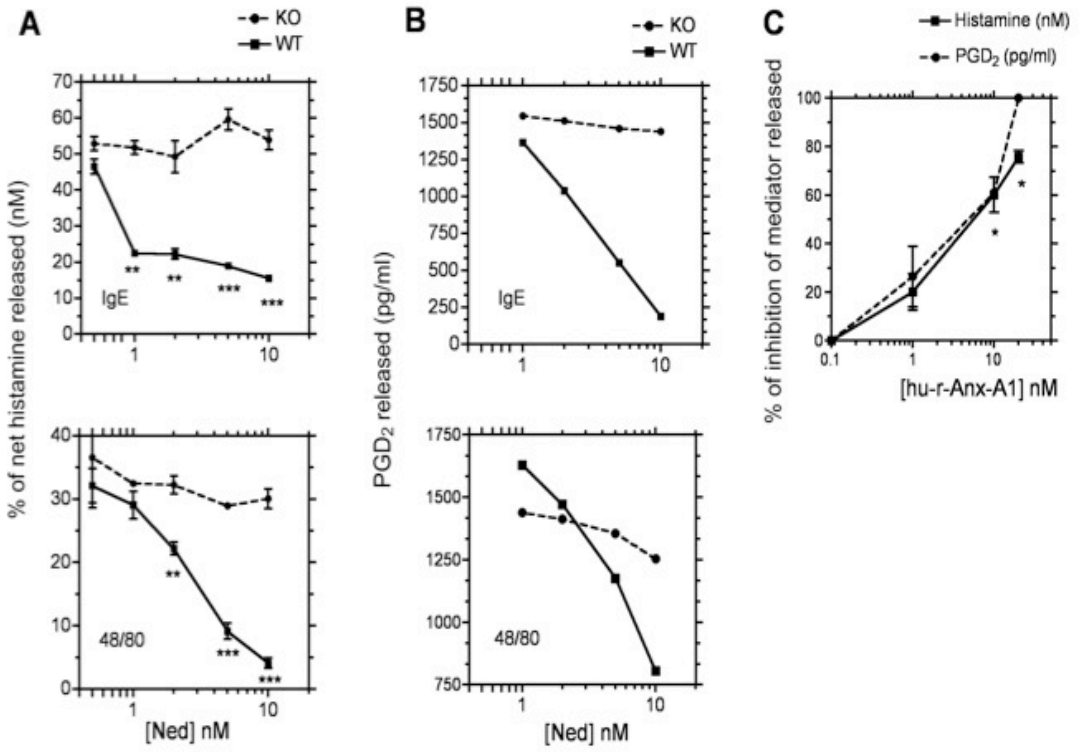


Figure 3.26: Nedocromil is inactive but hr-Anx-A1 inhibits PGD_2 and histamine in $Anx^{-/-}$ BMDMCs.

Panel A and B: BMDMCs were prepared from Anx-$A1^{-/-}$ and wild type mice as described. Samples were stimulated with either DNP/anti DNP cross-linking or compound 48/80. Nedocromil was added in increasing concentrations of 0.05 – 10nM and the supernatant was assessed by ELISA for histamine (A) and PGD_2 (B) release. Panel C: BMDMCs from Anx-$A1^{-/-}$ were pre-treated with hr-Anx-A1 (0.1 – 20nM) prior to stimulating the cells with compound 48/80 (10µg/ml). Histamine and PGD_2 release were assessed by ELISA. Results were expressed as % of inhibition; mean ± SEM of n=3 independent experiments. (Yazid *et al.*, 2013)

3.4.4 Release of tryptase by compound 48/80 was reversed by nedocromil in an Anx-A1 dependent manner.

Another important mediator that is released during mast cell degranulation is tryptase enzyme. Since nedocromil inhibited the release of histamine and PGD_2 in an Anx-A1 dependent manner, CDMCs were utilised to investigate if the release of tryptase was similarly inhibited and whether Anx-A1 was in fact in tryptase granules. Thus, the confocal microscopy was performed to investigate whether there is any co-localisation between Anx-A1 and tryptase granules in the CDMCs with or without the stimulation with compound 48/80. The cells were stained with an antibody against tryptase (green) and Anx-A1 (red). The confocal micrograph of unstimulated CDMCs (Figure 3.27; panel A) clearly illustrates that tryptase and Anx-A1 in CDMCs are not co-localised and are seemingly present in different subcellular locations. However, upon stimulation with compound 48/80 (panel B), the cell undergoes degranulation and some degree of co-localisation between tryptase and Anx-A1 was observed, suggesting that Anx-A1 may be recruited to tryptase granules in the cytoplasm or at the site of release at the membrane.

Next, the inhibitory action on the release of tryptase in CDMCs upon stimulation was investigated. Western blot was performed on the lysates of CDMCs pre-treated for 5 min with nedocromil (10nM) prior to the compound 48/80 (20µg/ml). Based on the blots (Figure 3.28; A), upon stimulation,

CDMCs significantly ($p<0.01$) released approximately 40% of intracellular tryptase (relative to the unstimulated cells). However, in the presence of nedocromil prior to stimulation with compound 48/80, about 90.8 ± 0.9 % of intracellular tryptase was retained in the cells. Interestingly, incubation of CDMCs the with neutralising anti-Anx-A1 monoclonal antibody for 20 min, reversed the inhibitory effects of nedocromil, as evidenced by a decrease in intracellular tryptase to 49.7 ± 4.5%. The presence of neutralising anti-Anx-A1 monoclonal antibody in CDMCs also increases the release of tryptase as it has been previously demonstrated that the Anx-A1 KO mice undergoes spontaneous mast cell degranulation (Yazid *et al.*, 2013), suggesting the important role of Anx-A1 in regulating the activation of mast cells.

To accompany this data, the morphology of CDMCs treated under similar conditions using confocal microscopy was observed. Figure 3.28 (panel B) shows that in the unstimulated cells, intracellular tryptase was observed in the cytoplasm. However, upon activation, the mast cell features an obvious disruption to the cell structure, whereby the tryptase was released out of the cell. Pre-treatment of nedocromil (10nM) before mast cell stimulation resulted in tryptase being retained intracellularly. In the presence of neutralising anti-Anx-A1 monoclonal antibody, nedocromil was not able to inhibit the release of tryptase as it is evident from the micrograph that the cell undergoes degranulation.

To further investigation, the cells were stained with toluidine blue, which detects the metachromatic granules of the mast cells. Unstimulated

cells exhibit intracellular metachromatic granules (purple) with intact nucleus (blue). Upon stimulation with compound 48/80, fewer intact intracellular metachromatic granules and granule-plasma membrane fusions can be observed during exocytosis.

Nedocromil inhibited mast cell degranulation but in the presence of neutralising anti-Anx-A1 monoclonal antibody, this effect was abrogated. CDMCs incubated with neutralising anti-Anx-A1 antibody alone have fewer metachromatic granules when compared to the control group. This suggests that without Anx-A1, the CDMCs spontaneously degranulate, and does not respond to nedocromil.

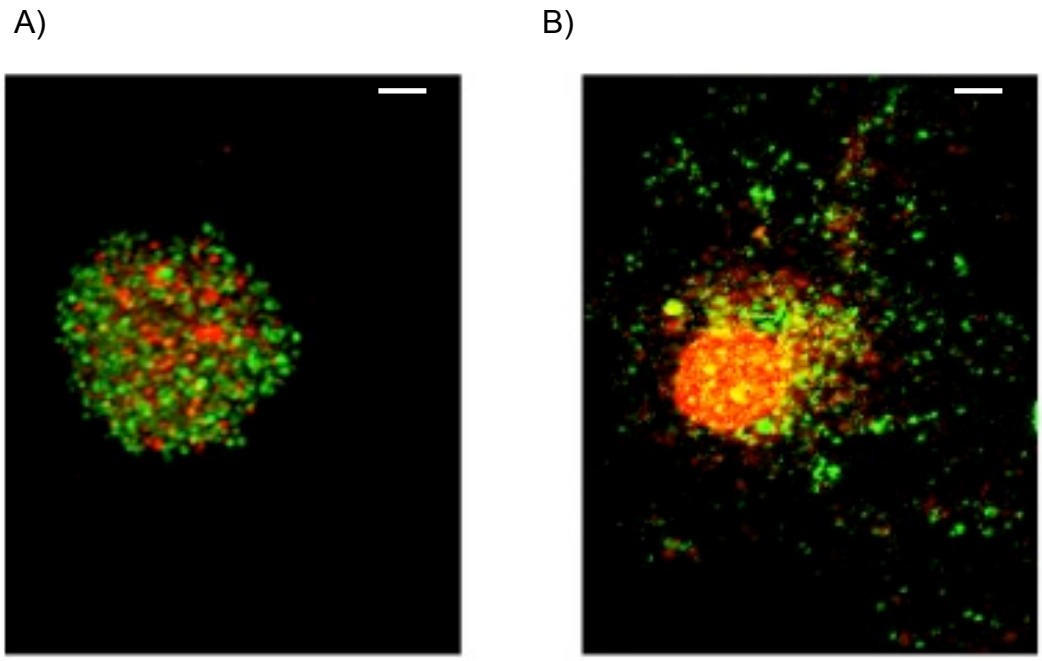


Figure 3.27: Localisation of tryptase and Anx-A1 in CDMCs.

Mast cells isolated from human cord-blood were stained with tryptase (1:1000 dilution) and Anx-A1 (1:2000) and were analysed by confocal immunofluorescence microscopy. Confocal images of unstimulated CDMCs (A) and stimulated with compound 48/80 (B) shows distinct degranulation of the mast cells in the presence of compound 48/80. These images clearly illustrate that tryptase (green) and Anx-A1 (red) in CDMCs are not co-localised as the tryptase and Anx-A1 do not overlap in resting cells. However, some degree of co-localisation was observed when the cells undergo degranulation. Pictures were taken at x63 oil magnification. These images are representative of 3 independent experiments. Scale bars indicate 10μm.

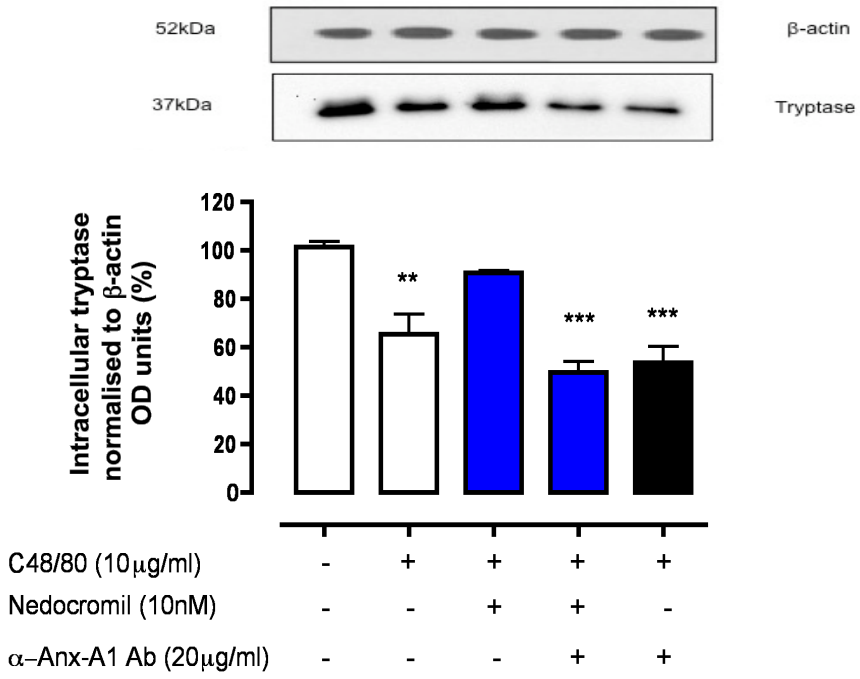
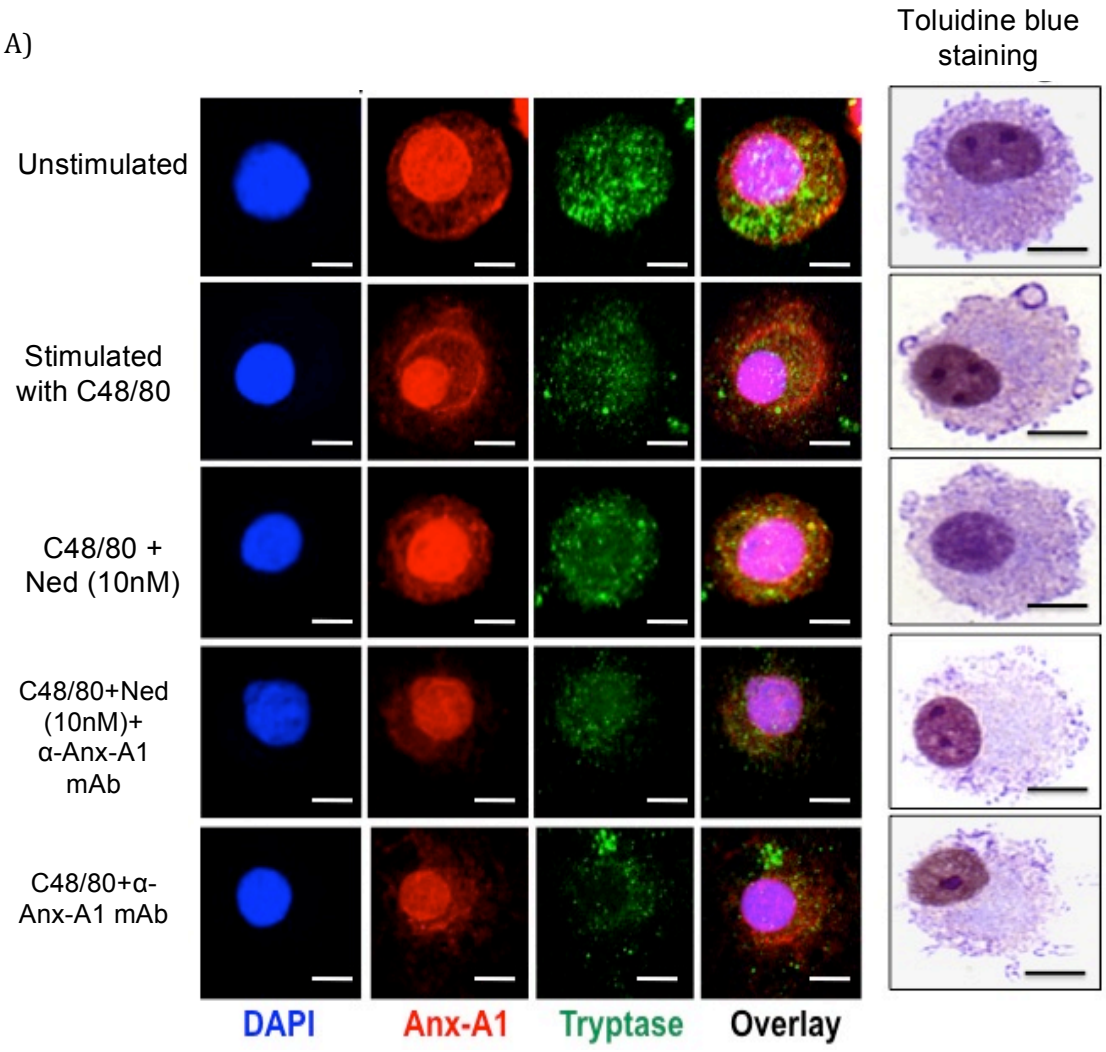


Figure 3.28: Western blot analysis of intracellular tryptase level in CDMCs.

The densitometry analysis on the Western blot shows that in the presence of degranulating stimuli, the tryptase level in the cell lysate is significantly reduced ($p<0.05$). Interestingly when the CDMCs were pre-treated with nedocromil (10nM), the tryptase remains in the cell. In the presence of the neutralising anti-Anx-A1 monoclonal antibody alone or combined with nedocromil, the tryptase level is significantly ($p<0.05$) reduced in the cell lysate. Data are expressed as mean ± SEM. (n=3 independent experiments, ** $p<0.01$, *** $p<0.001$ *vs* unstimulated).

A)


Toluidine blue staining
Unstimulated
Stimulated with C48/80
C48/80 + Ned (10nM)
C48/80+Ned (10nM)+ α-Anx-A1 mAb
C48/80+α-Anx-A1 mAb
DAPI
Anx-A1
Tryptase
Overlay

B)

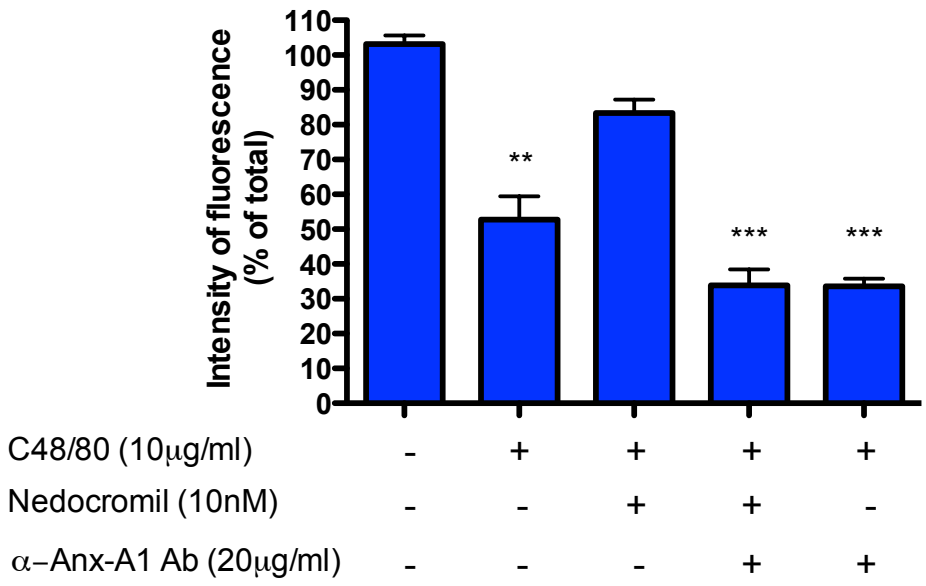


Figure 3.29: The inhibitory effect of nedocromil on tryptase release is Anx-A1 dependent in CDMCs.

A) Using CDMCs, confocal microscopy was utilised to visualise tryptase (green), total Anx-A1 (red) and DAPI was used to stain the nucleus (blue). CDMCs were stimulated with compound 48/80 (10μg/ml). Tryptase was confined in the cytoplasm when CDMCs were pre-treated with nedocromil prior to stimulation but in the presence of neutralising anti-Anx-A1 monoclonal antibody (20μg/ml), more tryptase granules were released from the cell. The morphology of the unstimulated mast cells shows that the plasma membrane is intact and the granules containing tryptase are contained inside the cell. However, upon activation, the mast cell exhibits obvious disruptions of the cell structure. This data was corroborated with toluidine blue staining of the cells treated under similar conditions observed under light microscopy. These images are representative of 3 independent experiments. Confocal images were taken at x63 oil magnification and the light microscopy images were obtained at x60 oil magnification. Scale bars indicate 10μm. B) The percentage of the intensity of fluorescence was calculated for each combination of treatment by voxel analysis of each confocal plane of >10 cells using Fiji image processing software (** $p<0.01$, *** $p<0.001$ *vs* unstimulated).

3.5 THE EFFECTS OF ANTI-ALLERGIC DRUGS ON THE INHIBITION OF HISTAMINE AND PGD_2.

3.5.1 Inhibition of histamine and PGD_2 release from CDMCs by nedocromil and ketotifen is mediated by Anx-A1, but promethazine exerts its anti-histamine effects independent of Anx-A1.

In previous experiments with CDMCs, nedocromil (Figure 3.14) and ketotifen (Figure 3.15) promoted Anx-A1 phosphorylation across various concentrations; however, promethazine (Figure 3.13) was not able to induce significant Anx-A1 phosphorylation in CDMCs. These drugs possess different pharmacological characteristics, in that promethazine is a H_1 antagonist, nedocromil is a mast cell stabilizer only and ketotifen is a 'dual-action' drug, having both properties. For this reason, the ability of these drugs to inhibit the release of mediators such as histamine and PGD_2 during mast cell degranulation and the role of Anx-A1 in their inhibitory effects was determined.

The drugs were prepared at a concentration of 10nM and incubated with the cells for 5 min prior to the compound 48/80 (10µg/ml) stimulation for 10 min. To ascertain the role of Anx-A1 in the mechanisms of action of these drugs, CDMCs were pre-incubated for 20 min with neutralising anti-Anx-A1 monoclonal antibody (20µg/ml), or irrelevant isotype matched antibody (20µg/ml) before proceeding with the drugs treatment. The supernatant was

collected and assayed for histamine or PGD_2. Figure 3.30 shows the effects of the co-incubation of CDMCs with neutralising anti-Anx-A1 (or irrelevant) monoclonal antibody on the effect of the inhibitory action of nedocromil, ketotifen and promethazine on histamine and PGD_2 release. Compound 48/80 stimulation provoked 45.9 ± 1.1 % of net histamine release and 1802 ± 199.2 pg/ml PGD_2 release. In the presence of neutralising anti-Anx-A1 monoclonal antibody, the release of histamine (58.3 ± 2.9%) and PGD_2 (2216 ± 164.3 pg/ml) was increased. Nedocromil significantly inhibited the release of histamine (20.8 ± 0.5%) and PGD_2 (743.9 ± 22.3pg/ml). However, in the presence of neutralising anti-Anx-A1 ab, nedocromil was not able to inhibit either the histamine or PGD_2 release. Similar effects were exerted by ketotifen, whereby, histamine release was significantly ($p<0.001$) inhibited by 19.1 ± 0.7% and PGD_2 release was significantly ($p < 0.001$) decreased by 508.5 ± 41.3 pg/ml. The ability of ketotifen to inhibit both histamine and PGD_2 release was abrogated in the presence of neutralising anti-Anx-A1 antibody, implying functional link between the action of this drug and the release of Anx-A1.

Promethazine did not inhibit the release of PGD_2 in stimulated CDMCs but was able to significantly ($p<0.05$) inhibit the release of histamine (27.5 ± 1.6%). However, in the presence of neutralising anti-Anx-A1 antibody, promethazine was still able to significantly ($p<0.05$) inhibit the release of histamine, suggesting that the mechanism of inhibitory action of

promethazine is not dependent on Anx-A1. Irrelevant isotype control was not active in this system.

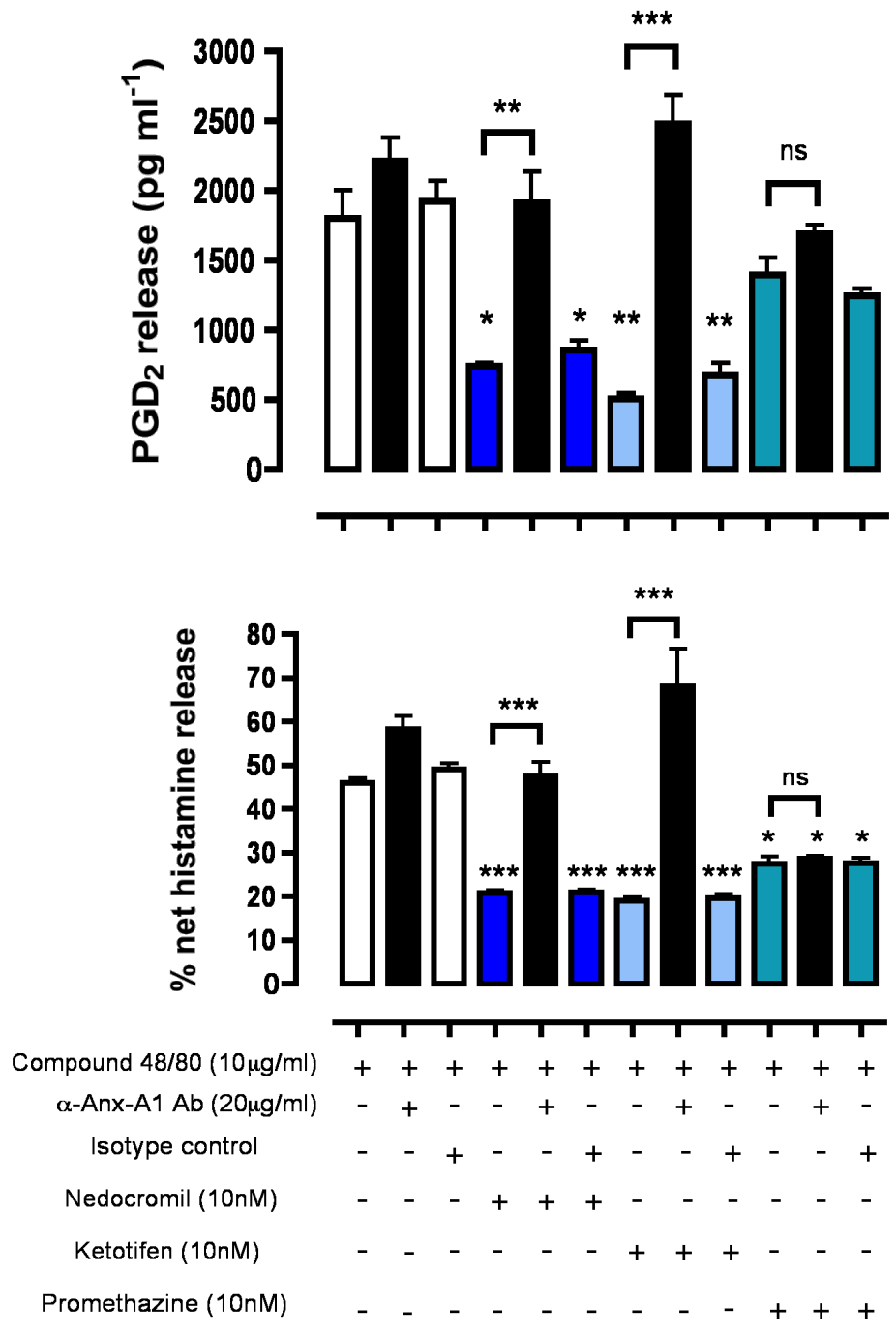


Figure 3.30: The inhibition of histamine and PGD_2 release by nedocromil and ketotifen is regulated by Anx-A1 but promethazine exerts its anti-histamine effects independent of Anx-A1.

CDMCs were cultured, pre-treated with the stipulated drugs and stimulated with compound 48/80 (10µg/ml) for 10 min as described. The cell culture supernatant was sampled and the PGD_2 (pg/ml) release and % net histamine release was assessed by ELISA. Data are expressed as mean ± SEM. (n=3 independent experiments, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* stimulated).

3.5.2 The effects of anti-allergic drugs on BMDMCs.

Nedocromil and ketotifen enhance the Anx-A1 phosphorylation in a concentration-dependent manner and these drugs inhibit the release of histamine and PGD_2 in an Anx-A1 dependent manner in CDMCs. However the H_1 antagonist, promethazine did not induce the phosphorylation of Anx-A1 and was only able to inhibit histamine in an Anx-A1 independent fashion. The reproducibility of this data was determined by performing similar experiment on the BMDMCs from both the WT and Anx-A1 KO strain.

Figure 3.31 (panel A) shows that compound 48/80-stimulated BMDMCs from WT mice released approximately 59.2 ± 0.7% of histamine and 628.4 ± 66.6 pg/ml of PGD_2. Similar to the previous experiment in CDMCs, nedocromil (10nM) significantly inhibited the release of histamine (25.3 ± 2.0%) and PGD_2 (244.8 ± 53.2pg/ml). Ketotifen (10nM) also significantly ($p<0.05$) inhibited both the release of histamine (48.3 ± 1.1%) and PGD_2 (263.3 ± 15.0pg/ml) in the WT BMDMCs. Promethazine (10nM) was not able to inhibit the release of either mediator from the WT BMDMCs.

Figure 3.31 (panel B) shows that compound 48/80-stimulated cells from the Anx-A1 KO mice released about 51.3 ± 0.5% of histamine and 652.1

± 19.9 pg/ml of PGD_2. Both nedocromil and ketotifen were inactive in the BMDMCs from the Anx-A1 null mice. The inhibitory action of the anti-inflammatory glucocorticoid dexamethasone (10nM), which is known to exert many effects through the release of Anx-A1 in these cells was tested. However, dexamethasone was without effect in the cells cultured from Anx-A1 null mice. Interestingly, promethazine was able significantly to inhibit both histamine (31.6 ± 1.9%) and PGD_2 (310.6 ± 35.1) in the KO BMDMCs, suggesting that promethazine is efficacious without Anx-A1. As observed previously, the human recombinant Anx-A1 was able to significantly ($p<0.001$) inhibit the release of both histamine (16.3 ± 0.2%) and PGD_2 (190.6 ± 39.1pg/ml) in these cells.

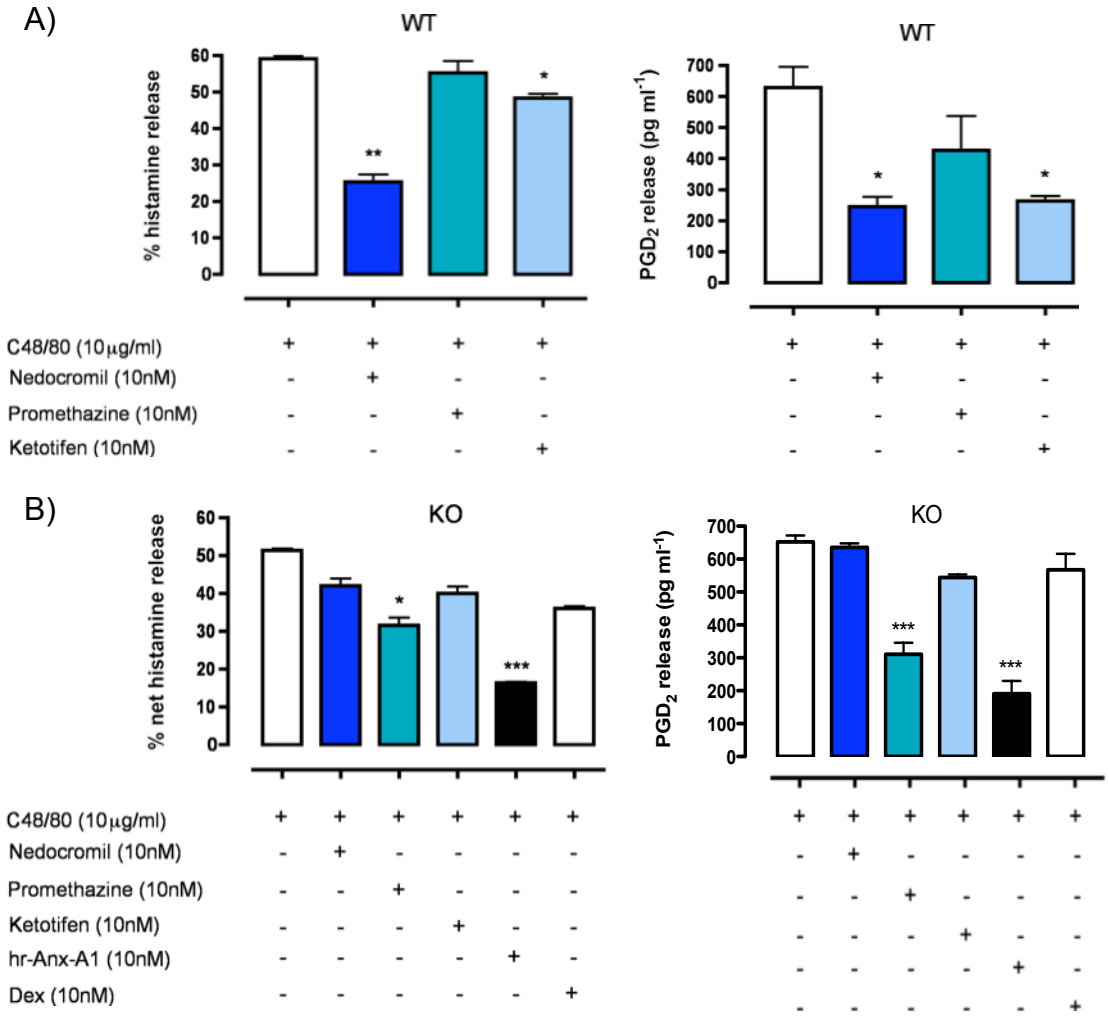


Figure 3.31: Nedocromil and ketotifen inhibit histamine and PGD_2 in WT BMDMCs but were not active in the Anx-A1 null cells. Promethazine was not active in the WT cells but was able to inhibit both mediators in the Anx-A1 null BMDMCs.

BMDMCs from the WT and Anx-A1 KO mice were cultured and pre-treated with the drugs prior to the stimulation with compound 48/80 (10µg/ml) before being assessed for histamine and PGD_2 release. Nedocromil and ketotifen inhibit the release of both the mediators in the WT BMDMCs but these drugs were inactive in the BMDMCs from KO mice. However promethazine inhibited both histamine and PGD_2 release in

the KO BMDMCs but not in the WT BMDMCs. Data are expressed as mean ± SEM. (n=3; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* stimulated).

3.6 THE ROLE OF FPR2 IN THE MECHANISMS OF MAST CELL DEGRANULATION BY NEDOCROMIL.

Formyl-peptide receptor 2 (FPR2) has been shown to transduce many biological activities of Anx-A1. Since it has been demonstrated in the previous experiments that Anx-A1 mediates the anti-allergic activity of nedocromil in mast cells, the possibility that FPR2 plays any significant role in the inhibition of histamine and PGD_2 release by nedocromil in CDMCs was determined.

To address this question, FPR2 antagonist, WRW4 peptide (10μm) was utilised. Figure 3.32 (panel A) indicates that the inhibition of PGD_2 release by nedocromil (520.9 ± 29.4 pg/ml) was reversed in the presence of WRW4 peptide, suggesting that nedocromil depends on the presence of FPR2 to exert its inhibitory effects. In contrast (panel B), the FPR2 antagonist did not prevent nedocromil (10nM) from inhibiting the release of histamine (20.8 ± 1.3%) in CDMCs, indicating that the acute inhibition of histamine by nedocromil is not dependent on FPR2. An interesting observation was that CDMCs incubated with WRW4 peptide alone, seems to have some inhibitory effects ($p<0.05$) on histamine release.

To provide additional data on the role of FPR2 in the mechanism of action of nedocromil, the BMDMCs from WT and fpr2/3 null mice were utilised. The cells were pre-treated for 5 min with a range of concentrations of nedocromil (0 - 50nM) prior to the stimulation of compound 48/80 (10μg/ml)

for 10 min. The supernatant from these cells were collected and assayed for either histamine or PGD_2 readouts. To measure the net level of histamine release, the spontaneous release of histamine from unstimulated cells were subtracted from the stimulated cells. The basal level of histamine is 57.1 ± 2.7% in the stimulated BMDMCs from the WT and 75.4 ± 12.3% in the BMDMCs retrieved from the fpr2/3 null mice. The levels of PGD_2 in stimulated WT BMDMCs were 1301 ± 601.4 pg/ml and 1806 ± 56.7 pg/ml in the fpr2/3 KO BMDMCs. Figure 3.33 (panel A) shows that nedocromil inhibited the release of PGD_2 in a concentration-dependent manner in the WT BMDMCs with an approximate IC_{50} value of 10nM, but nedocromil did not inhibit PGD_2 in the fpr2/3 KO BMDMCs. However, nedocromil exert a concomitant reduction in histamine levels across increasing concentrations in the BMDMCs from both the WT and the fpr2/3 null mice (panel B).

Collectively data from these two different cell types, CDMCs and BMDMCs, suggest that the inhibition of PGD_2 release by nedocromil is mediated by the release of Anx-A1 acting on FPR2, however, even though nedocromil inhibits the release of histamine by releasing Anx-A1, this mechanism does not seem to act through FPR2 but could be interacting with another member of the FPR family.

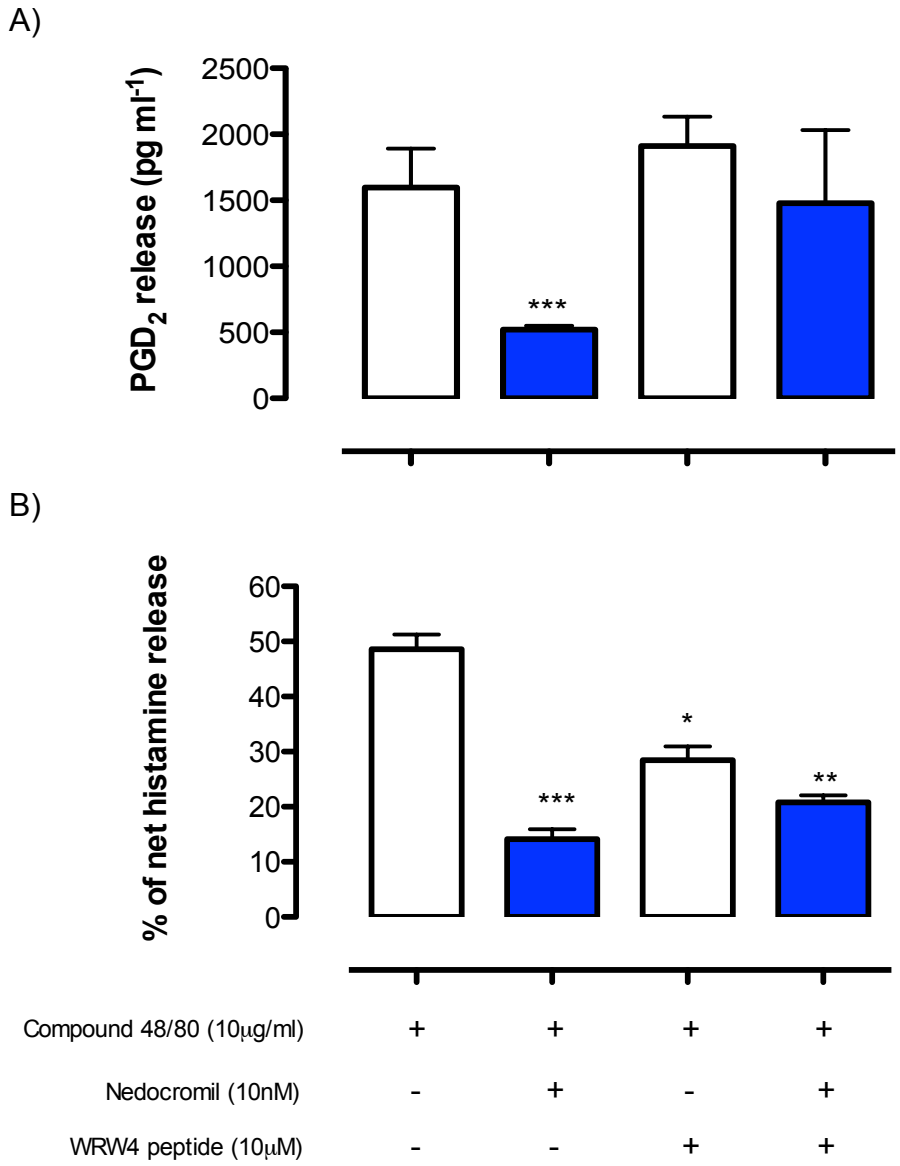


Figure 3.32: Inhibition by nedocromil (10nM) of PGD_2 but not histamine is FPR2 dependent in CDMCs.

The FPR2 antagonist (WRW4 peptide) at 10μM reversed the PGD_2 inhibitory effects of nedocromil, but was without effect in the inhibition of histamine suggesting that the inhibition of PGD_2 by nedocromil depends on the FPR2 pathway. Data are expressed as mean ± SEM. (n=3 independent experiments, ** $p<0.01$, *** $p<0.001$ *vs* stimulated).

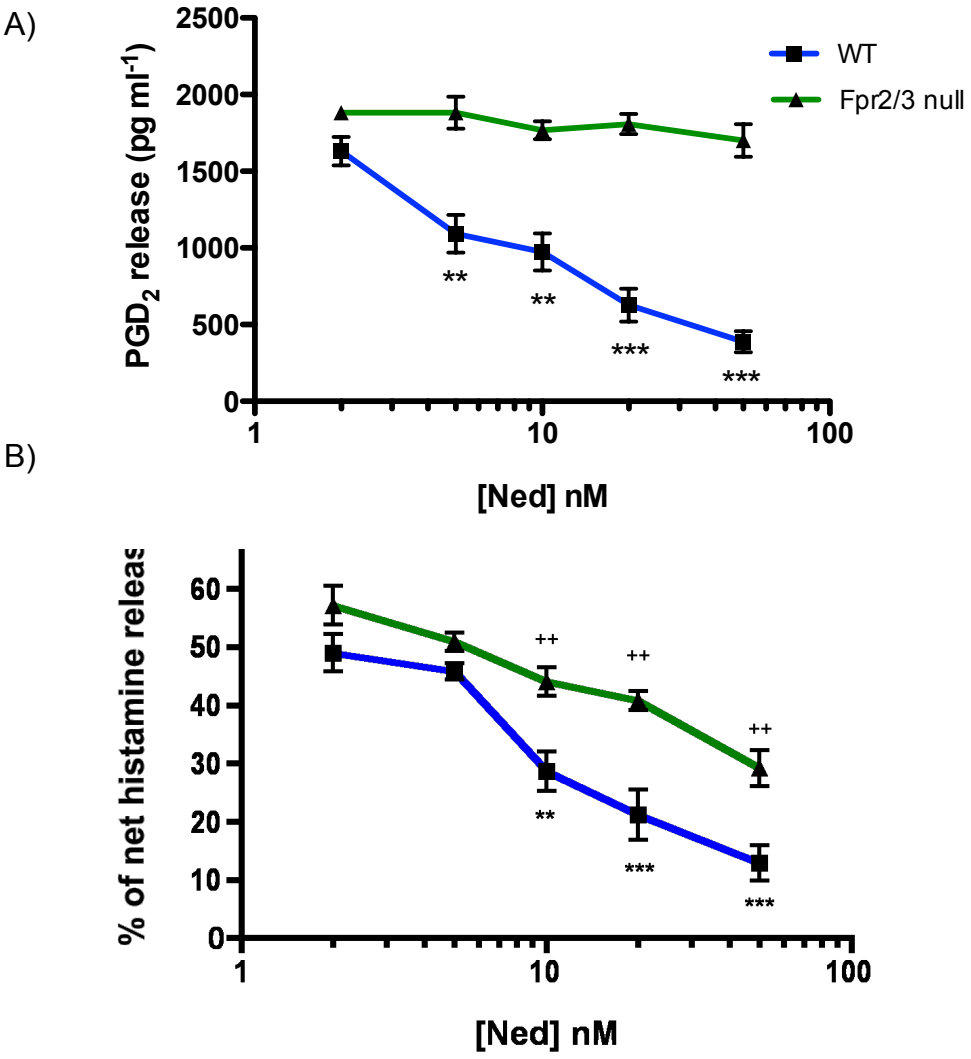


Figure 3.33: Nedocromil inhibits both histamine and PGD_2 in a concentration-dependent manner in the WT BMDMCs whilst in the fpr2/3 KO BMDMCs, nedocromil inhibits histamine but not PGD_2 release.

Nedocromil significantly inhibits the release of PGD_2 in the WT BMDMCs but not in the BMDMCs from fpr2/3 KO mice. However, nedocromil was able to significantly inhibit the release of histamine in a concentration dependent manner in BMDMCs from both the WT and fpr2/3 KO mice. Data are expressed as mean ± SEM. (n=3 independent experiments, ** $p<0.01$, *** $p<0.001$ *vs* stimulated).

3.7 NEDOCROMIL DOWN-REGULATES THE ACTIVATION OF p38 AND JNK IN CDMCS STIMULATED WITH COMPOUND 48/80.

GCs down-regulate mast cell activation by inducing dephosphorylation of protein kinases involved in the inflammatory signal transduction pathway (Oppong *et al.*, 2013). In line with this thought, a question that arised was if nedocromil promotes mast cell stabilization by altering the down-stream signaling of MAPK pathway, particularly p38 and pJNK in stimulated CDMCs.

To proceed with this experiment, compound 48/80-stimulated (10µg/ml) CDMCs pre-treated with nedocromil (10nM) was utilised. To determine the mechanism of nedocromil inhibition, neutralising anti-Anx-A1 monoclonal antibody (20µg/ml) was used. The lysates were analysed by Western blot using phospho-specific antibodies to p38 and pJNK. As a control, the levels of total kinases were also determined.

The densitometry analysis of the blot in Figure 3.34 indicates that compound 48/80 significantly ($p<0.001$) increased p38 phosphorylation (265.5 ± 8.3%) in CDMCs in comparison with the unstimulated cells. However, nedocromil inhibited p38 phosphorylation in stimulated CDMCs. In the presence of neutralising anti-Anx-A1 antibody, nedocromil did not down-regulate the activation of p38, suggesting that Anx-A1 indeed plays a role in the abrogation of p38 phosphorylation by nedocromil.

Figure 3.35 shows that compound 48/80-stimulated CDMCs significantly ($p<0.001$) induced JNK phosphorylation by 245.5 ± 3.5% in

comparison with the unstimulated cells. Nedocromil inhibited the phosphorylation of JNK, however immuno-neutralisation of Anx-A1 in CDMCs was without effect. Even though only a small component of down-stream signaling pathway was tested in this study, the mechanistic signaling signature of nedocromil could be identified, where by Anx-A1 plays a differential role in the nedocromil-induced down-regulation of p38 and JNK activation in mast cells.

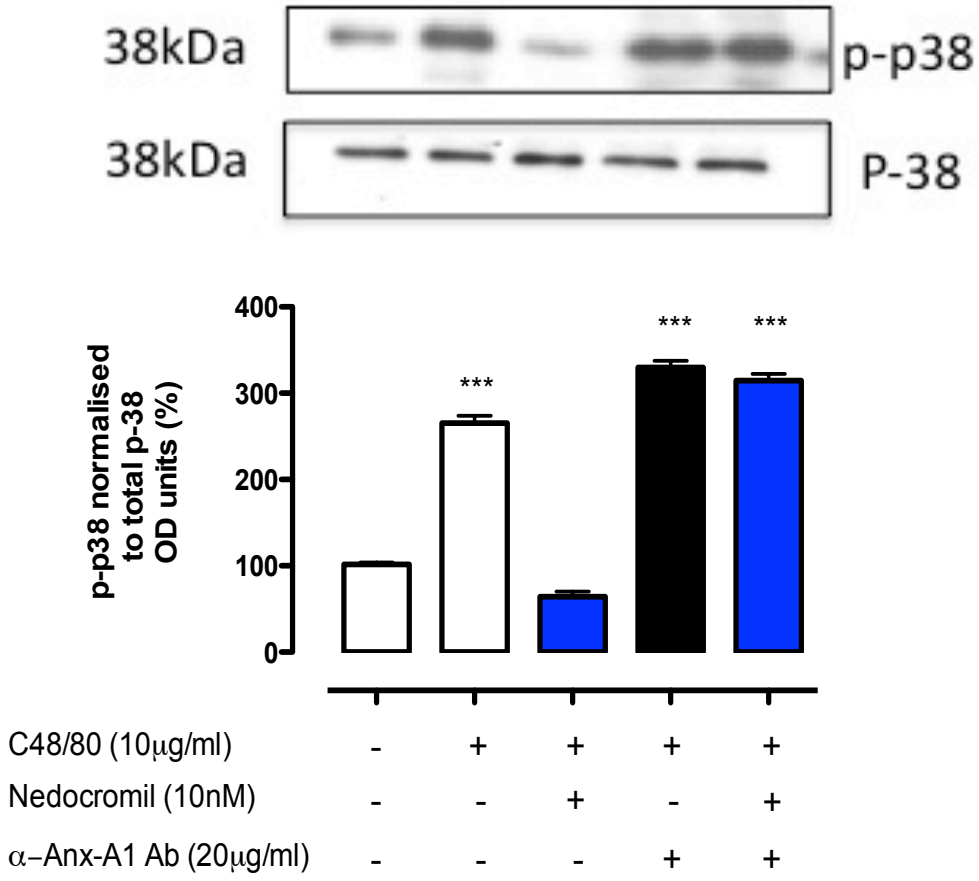


Figure 3.34: Compound 48/80-induced p-38 phosphorylation was reduced in CDMCs pre-treated with nedocromil, but not when Anx-A1 is immuno-neutralised.

Compound 48/80 stimulation of CDMCs increased p38 phosphorylation, however this effect was inhibited by nedocromil (10nM). Neutralising anti-Anx-A1 monoconal antibody (20μg/ml) reversed the phospho-p38 inhibitory actions of nedocromil. Data are expressed as mean ± SEM. (n=3 independent experiments; *** $p<0.001$ *vs* unstimulated).

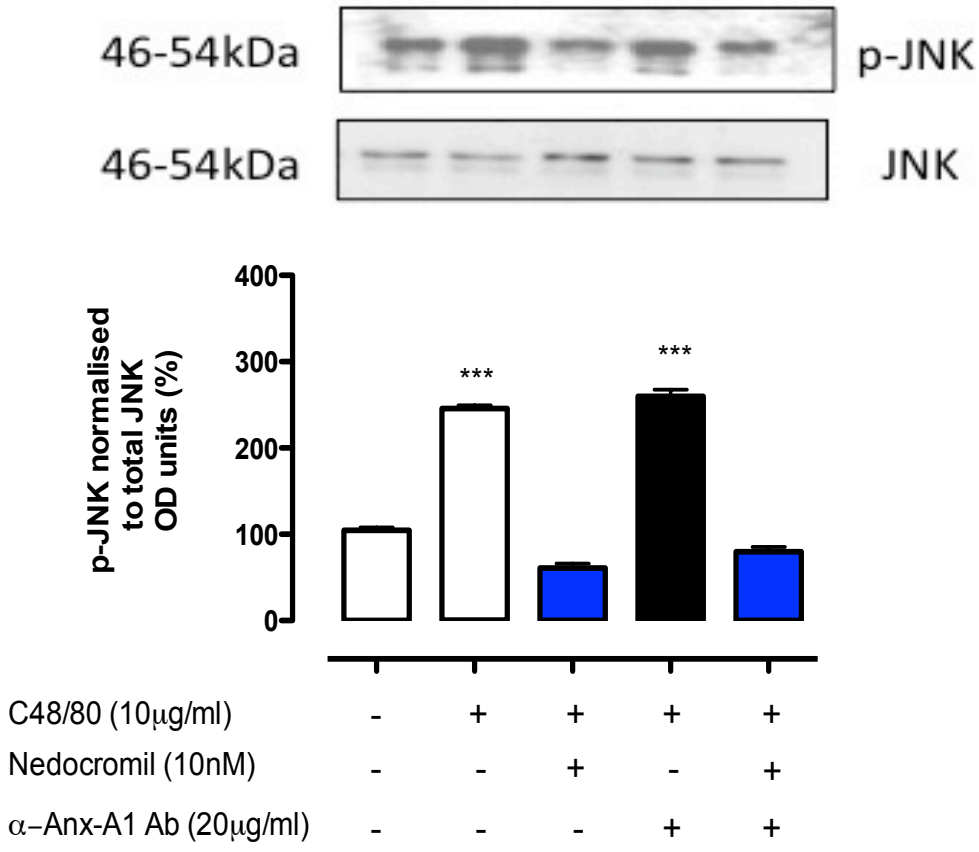


Figure 3.35: Nedocromil inhibits JNK phosphorylation in stimulated CDMCs in an Anx-A1 independent manner.

Densitometry analysis shows that compound 48/80-induced JNK phosphorylation was reduced in the presence of nedocromil (10nM). The presence of neutralising anti-Anx-A1 monoclonal antibody did not alter the inhibitory actions of nedocromil on JNK phosphorylation. Data are expressed as mean ± SEM. (n=3 independent experiments; *** $p<0.001$ *vs* unstimulated).

4. DISCUSSION

The anti-allergic cromones were discovered over five decades ago, but their mechanism of action until recently, was very vague. This thesis seeks to understand the mechanisms of pharmacological action of these drugs on mast cells. Given that Anx-A1 is an important endogenous anti-inflammatory mediator, the fundamental mechanistic relationship between Anx-A1 and anti-allergic drugs in the mast cell model was addressed.

Anx-A1 is both induced and secreted by many differentiated cell types but is particularly abundant in neutrophils, eosinophils, macrophages and mast cells (Kamal *et al.*, 2005). Surprisingly though, the actual molecular mechanism of Anx-A1 secretion is not well understood. Since the protein does not contain a canonical signal sequence, a generally accepted notion is that secretion occurs through a non-classical pathway, and some evidence suggests that the ABC transporters may be involved (Wein *et al.*, 2004) or that the ceramide platform in cells could preferentially bind Anx-A1 and promote externalisation (Babiychuk *et al.*, 2008). GCs induce Anx-A1 release via a cascade of signalling events (Solito *et al.*, 2003c; John *et al.*, 2004), in which activation of PKC triggers Anx-A1 phosphorylation at Ser^{27} residue and possibly other sites prior to externalisation (Solito *et al.*, 2006b).

In this thesis, a possible mechanistic explanation for the acute pharmacological actions of cromoglycate-like drugs and other anti-allergic drugs is proposed which may shed some light on a new pathway whereby the release of mediators from mast cells could be modulated.

4.1 Mechanism of action of anti-allergic drugs in U937 cells.

Because Anx-A1 export is a prerequisite for its extracellular anti-inflammatory activities, it was deemed important to characterise the ability of these drugs to promote the phosphorylation of Anx-A1. Incidentally, GCs promote tyrosine phosphorylation of Anx-A1 in the human lung carcinoma cells (A549 cell line) (Croxtall *et al.*, 2002), a finding that contrasts with another study performed on human folliculostellate cells (Solito *et al.*, 2003c), raising the possibility that GCs promote phosphorylation of Anx-A1 in a cell- and tissue-specific manner.

GCs have a dual effect on Anx-A1 disposition, in that they not only potentiate rapid non-genomic action (Croxtall *et al.*, 2000) but also increase Anx-A1 mRNA, or at least in U937 cells (Solito *et al.*, 1992). In this thesis, the non-genomic effects of these drugs were focused upon.

To assess the ability of anti-allergic drugs to stimulate phosphorylation of Anx-A1, the human pro-monocytic cell line, U937 was chosen as the first model because these cells respond well to GC stimulation and express significant amount of intracellular Anx-A1 protein. In line with the data obtained by other investigators, no effect of GCs was observed in the absence of PMA 'priming' for 24 h before treatment, as studies have revealed that dexamethasone-induced mRNA expression of Anx-A1 only occurs in differentiated U937 cells (Solito *et al.*, 1991a). The 5 min time point was chosen for the treatment of anti-allergic drugs on U937 cells based on previous reports which have identified that this time point to be optimal for

Anx-A1 phosphorylation in folliculostellate cells stimulated with LPS (Solito *et al.*, 2006b) and GCs treatment on U937 cells (Yazid *et al.*, 2009).

Evidence from the biochemical and imaging studies performed here has shown that 'pure' H_1 antagonist drugs have only weak effects on Anx-A1 phosphorylation and release. The 'mast cell stabilising' drugs used in this study have differing abilities to promote Anx-A1 phosphorylation. Cromoglycate and pemirolast have a moderate ability to promote Anx-A1 phosphorylation. However, nedocromil greatly enhanced Anx-A1 phosphorylation. Congruent with its clinical efficacy, it is noteworthy that nedocromil is 10 times more potent than cromoglycate in this respect (Yazid *et al.*, 2009). Although cromoglycate is less effective as a mast cell stabiliser than nedocromil in our system, it has more clinical efficacy in asthma and allergic conjunctivitis. Whilst there are apparent differences between these drugs in clinical efficacy or potency *in-vitro*, it could be interpreted that this could be due to the differences in pharmacokinetics and biodistribution of these drugs. It is argueable that the low concentrations used in the *in-vitro* assays may suggest that the therapeutic benefit is not related to the mechanisms described here, however, it is important to note that these drugs are clinically used as inhalers or eye drops, and thus the dosing reflects on the fraction of drug that is absorbed into the body.

The cromones have distinct pharmacological actions (Cox, 1967; Cox, 1970; Cox *et al.*, 1970) in that other 'mast cell stabilising' drugs such as β agonists do not share the same characteristic gamut of activity of the

cromones (Shichijo *et al.*, 1998). Of the 'dual-action drugs, olopatidine only moderately enhances Anx-A1 phosphorylation, whereas ketotifen and epinastine greatly enhanced this effect.

Since these drugs seem to act by promoting the release of Anx-A1, their eicosanoid inhibitory activities were investigated. Congruent with past studies (Yazid *et al.*, 2009), thromboxane (Tx) B_2 inhibition by nedocromil was greatly potentiated by low concentrations of dexamethasone. A speculation would be that nedocromil accomplishes this mechanism by prolonging PKC activation at the plasma membrane, thereby potentiating Anx-A1 phosphorylation and release, but only when the cells were primed by dexamethasone or other PKC activating stimulus. Indeed, the use of a specific neutralising anti-Anx-A1 monoclonal antibody has supported this hypothesis and confirmed earlier reports that TxB_2 inhibitory effect is mediated by Anx-A1 (Solito *et al.*, 1991a; Solito *et al.*, 1993).

4.2 The role of PKC in Anx-A1 export in mast cells.

PKC is a family of isoenzymes that undergoes translocation from one subcellular compartment to another in response to extracellular stimulation (Yamaguchi *et al.*, 1995). When activated by diacylglycerol (DAG), phospholipids or calcium, PKC is phosphorylated first at Ser^{657} residue, which controls the accumulation of phosphate at other sites and contributes to its phosphatase resistance conformation (Bornancin *et al.*, 1997). These post-translational modifications convert the soluble inactive form to an active

enzyme that translocates from the cytoplasm to the plasma membrane, possibly mediated by the receptors for activated-C kinases (RACKs). It is at the membrane that the enzyme principally phosphorylates its target substrates (Mochly-Rosen *et al.*, 1991). The classical or conventional PKC isozymes (cPKCs; α, βI, βII, γ) are Ca^{2+}-dependent, activated by DAG and bind to negatively charged membrane phospholipids, in particular phosphatidylserine (PS). Novel PKCs (nPKCs; ε, δ, θ, η) are Ca^{2+}-independent and only need DAG and PS for activation, whereas the atypical PKCs (aPKCs; ζ, λ/ι) require neither Ca^{2+} nor DAG, but can be activated by PS (Hoque *et al*., 2014).

Investigation of the mechanism by which PKC activity is terminated by dephosphorylation at the membrane was beyond the scope of this thesis, however previous work from several authors suggest that this was caused by the Ser/Thr phosphatase PP2A followed by ubiquitination (Hansra *et al.*, 1996; Boudreau *et al.*, 2002; Lee *et al.*, 2008). PP2A is a heterotrimeric enzyme, which comprises one each of two catalytic and structural subunits together with one modulatory or targeting subunit. PP2A undergoes post-translational modifications, such as phosphorylation and methylation, which in turn, regulate its enzymatic activity. It plays a critical role in cellular processes, such as cell proliferation, signal transduction and apoptosis (Lambrecht *et al.*, 2013). In many cell types, including macrophages, PKCα is controlled by the activity of the phosphatase, which limits the catalytic action of the kinase by dephosphorylation (Ricciarelli *et al.*, 1998; Boudreau *et al.*,

2002). Moreover, an elegant study has demonstrated the physical association of PKC and PP2A in resting mast cells, which confirms a functional interaction between these two enzymes (Boudreau *et al.*, 2002). It has been reported previously in U937 cells that the degree of Anx-A1 phosphorylation, hence the amount exported, will depend on the net catalytic activity of the PKC-PP2A complex, which in turn results from the reciprocal interactions between the two enzymes (Yazid *et al.*, 2009).

Even though much work has been done on the effect of cromoglycate-like drugs on the GC-Anx-A1 system in the U937 cells (Yazid *et al.*, 2009), the main target for the cromones and the anti-allergic drugs are the mast cells. Thus, the majority part of this thesis was focused on work performed on cord-blood derived mast cells (CDMCs) and bone marrow derived murine mast cells (BMDMCs).

The results described here confirmed previous reports (Solito *et al.*, 2003b; Solito *et al.*, 2006b) that in these cells, Anx-A1 translocation from the cytoplasm on to the cell surface is preceded by the phosphorylation of Anx-A1 and established that the kinase described is PKC, more specifically the α/β isoenzymes (Yazid *et al.*, 2013).

The time course of nedocromil action was monitored and revealed that 5 min was the most optimal time point to record maximal PKC and Ser^{27}-Anx-A1-P in CDMCs. This time point is identical to the time course of LPS action on Anx-A1 phosphorylation in the folliculostellate cells (Solito *et al.*, 2006a) and GC-induced Anx-A1 phosphorylation in the U937 cells (Yazid *et al.*,

2009). Therefore, 5 min time point was chosen to treat the CDMCs with anti-allergic drugs to monitor their ability to exert acute effects.

This data here clearly indicates that nedocromil, prolongs PKC activation and subsequently phosphorylation, externalization and release of Anx-A1 from CDMCs. Other anti-allergic drugs such as ketotifen and promethazine were also tested in this model and the results show that whilst ketotifen promotes Anx-A1 and PKC phosphorylation in a concentration-dependent manner, promethazine was not active in inducing Anx-A1 phosphorylation in CDMCs. It is noteworthy to mention that the anti-allergic drug ketotifen, a non-cromone member appears to have similar actions on the Anx-A1 system. Ketotifen is a H_1 antagonist that has long been observed to have additional 'mast cell stabilising' properties (Cook *et al.*, 2002).

Though the inhibition of PP2A by anti-allergic drugs is not directly investigated in this study, an indirect way was used to show that the cromoglycate-like drugs prolong the duration of PKC activation by GCs. The Western blot analysis demonstrated that the treatment of CDMCs with a combination of both dexamethasone and nedocromil synergistically potentiated the PKC phosphorylation with time when compared with dexamethasone alone, suggesting that indeed nedocromil is able to prolong the 'dwell' time of PKC activation, perhaps by limiting the catalytic action of the PP2A enzyme. To further study the ability of dexamethasone and nedocromil to mobilise PKC in CDMCs, confocal microscopy was performed using fluorescent-labelled antibody against PKC. The micrographs show that

PKC has translocated to the plasma membrane upon treatment with dexamethasone for 5 min, however this effect was not maintained across time. This observation indicates that the phosphatase enzyme at the plasma membrane has terminated the PKC activation. Interestingly, when CDMCs were treated with dexamethasone and nedocromil simultaneously, the PKC 'dwelling' time at the plasma membrane is prolonged and is still detectable at 60 min, supporting the notion that this effect is brought about following inhibition of cromoglycate-like drugs of a phosphatase, probably PP2A, which secondarily prolongs the activation of PKC thereby further stimulating Anx-A1 phosphorylation and release.

There have been scattered reports of an interaction between cromoglycate and PKC stretching back over some years. Indeed, there has been a study that investigated the possibility that these drugs inhibited PP2A, but were unable to detect an effect in their system (Wang *et al.*, 1999). Other reports have a correlation between the action of cromoglycate-like drugs *in vivo* in a rat passive cutaneous anaphylaxis model and inhibition of another, alkaline phosphatase (Schwender, 1981; Schwender *et al.*, 1982; Ye *et al.*, 2009).

4.3 The possibility that the mechanisms of action of some anti-allergic drugs are mediated by Anx-A1 in mast cells.

Evidence from biochemical and imaging studies described here have further confirmed that the ability of these anti-allergic drugs to release Anx-A1 is critical for their acute mediator inhibitory actions in CDMCs and BMDMCs. This mechanism is common to several types of cells as was demonstrated in previous observations using U937 cells (Yazid *et al.*, 2009) and PMN (Yazid *et al.*, 2010b), where a similar autocrine Anx-A1 dependent inhibition of cell function was observed following cromone treatment. It has been reported that dexamethasone, a GC known to release Anx-A1 using this pathway (D'Acquisto *et al.*, 2008), also inhibits histamine and PGD_2 release in these cells (Yazid *et al.*, 2013).

For this study, compound 48/80 was utilised as a stimulant to activate the mast cells. Extensive literature is available on the IgE mediated mechanism of mast cell activation, but the mechanisms by which cromoglycate-like drugs inhibit compound 48/80-induced mast cell degranulation is rather scarce. Thus, this led to the investigation of the effects of cromoglycate-like drugs and other anti-allergic drugs on CDMCs and BMDMCs stimulated by compound 48/80. This mast cell secretagogue activates mast cell secretory processes by increasing the rate of GTPγS binding to G-proteins. This eventually triggers intracellular signaling events such as activation of phospholipase D (PLD), protein kinase C (PKC) and

Ca^{2+} signaling which ultimately results in mast cell degranulation (Palomäki *et al.*, 2006). This data indicates that compound 48/80 induces the release of histamine across increasing concentrations in CDMCs, and similar trend was reported in mast cells purified from the peritoneal cavity of male Wistar rats (Aridor *et al.*, 1990).

Since compound 48/80 activates intracellular signaling, which culminates in PKC signaling, the ability of the secretagogue to promote phosphorylation of Anx-A1 itself was analysed. Another important observation noted was that Anx-A1 phosphorylation induced by compound 48/80 was short-lived, decaying after 5 min, however nedocromil was able to prolong Anx-A1 phosphorylation to 40 min in CDMCs stimulated by compound 48/80. This suggest that nedocromil exerts its 'mast cell stabilising' effects in stimulated cells by enhancing the phosphorylation of Anx-A1 and therefore the export of the protein out of the cell. Even though both the compound 48/80 and cromoglycate-like drugs triggers PKC activation, the downstream activation undergoes a divergent pathway, which results in different outcomes. This is probably because compound 48/80 triggers a different PKC isoform as opposed to the cromones.

Based on the Western blots data, it is important to note that there is a distinct difference between the ability of the anti-allergic drugs to enhance Anx-A1 phosphorylation in the U937 cells or CDMCs. Ketotifen and nedocromil potentiated Anx-A1 phosphorylation in a concentration-dependent manner in the U937 cells, however in the CDMCs, there was bell shaped

curve across increasing concentrations. A possible explanation for this phenomenon could be that U937 cells were pre-treated with PMA, which is an activator of PKC, thus the cells are already 'primed' and there is already a small pool of phosphorylated intracellular Anx-A1 present in these cells even before drug treatment leading to a potentiation at higher concentration of drugs. Interestingly, promethazine, which is a H_1 antagonist drug, does not potentiate Anx-A1 phosphorylation in either U937 cells or CDMCs, although it has been shown elsewhere that promethazine, a phenothiazine-derived drug is able to interact with Anx-A1 in neutrophils (Blackwood *et al.*, 1995).

The data further suggest that Anx-A1 plays an important role in 'stabilising' mast cells as unstimulated CDMCs undergo spontaneous degranulation indicated by β-hexosaminidase release when Anx-A1 is depleted by immune-neutralisation. Although the release of β-hexosaminidase is a widely used indicator of secretory lysosome exocytosis from mast cells, it is unclear whether the release of this single granule marker accurately reflects the behavior of all secretory granules in mast cells. Thus, in addition to β-hexosaminidase, the inhibition of tryptase, histamine and PGD_2 release by the anti-allergic drugs were measured. An interesting observation was noted in the model of 'seasonal allergic conjunctivitis' (SAC), whereby the Anx-A1 KO mice display a more pronounced allergic conjunctivitis after sensitisation with ragweed pollen when compared to wild type counterparts, further reinforcing the role of Anx-A1 in regulating mast cell stability (Yazid *et al*., 2014 unpublished).

To determine whether tryptase and Anx-A1 are present in the same granules or whether there is heterogeneity within the secretory granule population of mast cells, immunofluorescence techniques were used. Observation from the confocal images shows that the distribution of tryptase and Anx-A1 are present in different granules, as they don't co-localise in resting cells. The Western blot and immunofluorescence data clearly indicates that compound 48/80-stimulated CDMCs release tryptase into the extracellular milieu, but nedocromil inhibits the release of tryptase in an Anx-A1 dependent manner. This data is in agreement with previous reports, which suggest that nedocromil inhibits the release of tryptase as part of its 'mast cell stabilising' effects (Calhoun *et al.*, 1996; Kempuraj *et al.*, 2005), though the results presented here shows a novel finding that Anx-A1 is indeed responsible for the acute inhibitory effects of nedocromil on tryptase release. The data has therefore clearly shown that not only is Anx-A1 exported out of the mast cells upon treatment of cromones but is also thought to be critical for the inhibition of mediator release from the mast cells.

The amounts of Anx-A1 released from CDMCs treated with nedocromil and ketotifen, yields concentrations in the nM range, that are substantial enough to reduce histamine and PGD_2, as indicated by the experiments with recombinant human Anx-A1. This effect is brought about by Anx-A1, since both the drugs were unable to inhibit the mediator release as in the BMDMCs isolated from Anx-A1 null mice. (Yazid, Sinniah *et al.*, 2013). This effect of eicosanoid release and inflammation was also observed in the Anx-$A1^{-/-}$ null

mice in which dexamethasone was shown to be inactive (Croxtall *et al.*, 2003). These experiments have clearly demonstrated that nedocromil and ketotifen are dependent on Anx-A1 release to exert its anti-inflammatory effects. Since ketotifen exerts similar mechanism of action to nedocromil, it is interesting to speculate that all the H_1 antagonists, which are also mast cell stabilisers, that have this additional action, may have a secondary pharmacology as PP2A inhibitors and that this could be a useful screen to evaluate this drug's property.

The action of promethazine differs from nedocromil and ketotifen. At 10nM, the drug was not able to inhibit the release of PGD_2 from activated mast cells, but significantly inhibited the release of histamine from CDMCs. But immuno-neutralisation techniques in these cells have shown that the inhibitory action of promethazine is not mediated by Anx-A1. When similar experiments were repeated on the WT BMDMCs, promethazine did not inhibit the release of both histamine and PGD_2, which would imply a considerable species-variation in the response to this drug. However, promethazine significantly inhibited the mediator release in BMDMCs from Anx-A1 null mice, suggesting that promethazine only exerts its inhibitory effect in the absence of Anx-A1. A possible speculation for this anomalous observation could be that in the Anx-A1 null mice, the H_1 receptors are overexpressed, hence promethazine is able to function better in these mice.

It is important to note that the cromones can only exert appreciable effect on mediator release if Anx-A1 phosphorylation is already triggered by

another stimulus, such differences could reflect the relative degree of activation of cells from different sources or subjected to different experimental protocols. Perhaps this explains why the cromoglycate drugs have only a variable effect when given in the absence of an appropriate 'priming' stimulus that triggers this effect. This observation might also explain some of the anomalous dose-response relationships that have been reported (Church *et al.*, 1987; Okayama *et al.*, 1992b). In this context it is interesting to note that the CDMCs cultured as described here are partly activated by the presence of SCF and that there is already a small pool of intracellular Ser^{27} phospho Anx-A1 present in these cells even before challenge with antigen or drug treatment.

However there are some anomalous observations, which indicate that, cromones have failed to inhibit histamine release from anti-IgE stimulated human conjunctival mast cells (Canonica *et al.*, 1997) and human connective tissue type mast cells (Okayama *et al.*, 1992a; Okayama *et al.*, 1992b). A recent study has suggested that cromoglycate is not active at all in the mouse model (Oka *et al.*, 2012) although the results presented here show that nedocromil responds well in the BMDMCs model. This heterogeneity between species and different mast cell subtypes in response to the drugs is a conundrum. Contributing to this could be the timing and concentrations of cromones pre-treatment prior to mast cell degranulation, which were thought to relate to the differing sensitivity (Shichijo *et al.*, 1998). Another speculation

is that the existence of different Anx-A1 pools within mast cells from different sites (Oliani *et al.*, 2000) could explain the variation of the cromones effects.

The dependency of nedocromil on Anx-A1 for its anti inflammatory effect presented here is novel but there have been previous observations in the past suggesting that cromones trigger the phosphorylation of intracellular protein substrates including the erythrocyte band 4.1 group protein moesin, which leads to the modification of downstream mediators of molecular targets in mast cells (Theoharides *et al.*, 2000). Interestingly, cromones were also shown to have an interaction with PKC (Lucas *et al.*, 1987; Bansal *et al.*, 1997), which fits well in the hypothesis. Moreover, cromoglycate sodium, which inhibits IgE mediated mast cell degranulation has been shown to interact with S100-Ca^{2+} binding proteins which bind to Anx-A1 to Anx-A5 (Oyama *et al.*, 1997), suggesting a potential interaction between cromones and annexins.

There has been recent revival in cromone literature and it has also been suggested that cromones produce their therapeutic effects by interacting with GPR35 through the G_i pathway (Jenkins *et al.*, 2010; Yang *et al.*, 2010). Apparently GPR35 is present in human mast cells stimulated with IgE cross-linking, although it's significance to mast cell mediator release is not fully understood. How this notion could be integrated into Anx-A1 dependent mechanism is unclear, but it is worth noting that the effects of cromones that was detailed here occurred within 5 min and possibly at longer duration of cromone treatment the GPR35 mechanisms may be important.

4.4 The role of FPR2 in the inhibitory effects of mediator release by nedocromil.

Because there is compelling evidence to support the notion that nedocromil exerts its effect through the Anx-A1 pathway, the possibility that one of the principal receptor for this protein, FPR2 might also be involved in the mechanism of action of nedocromil was explored. This was a point of interest, since both Anx-A1 protein and the FPR2 are expressed in mast cells (Lee *et al.*, 1997; Oliani *et al.*, 2000). To address this aim, not only FPR2 antagonist, WRW4 peptide was utilised but also BMDMCs isolated from the fpr2/3 null mice colony was used to further ascertain the role of FPR2 in the acute effects of nedocromil.

The release of two mediators of mast cells, histamine and PGD_2 were tested. Based on the results obtained from CDMCs, nedocromil inhibits PGD_2 through the activation of FPR2, as in the presence of FPR2 antagonist the acute inhibitory effect of nedocromil was reversed. In contrast, inhibition of histamine release by nedocromil was not mediated by FPR2 activation since nedocromil was still able to significantly inhibit histamine release in the presence of WRW4 peptide. This observation reflects that even though the inhibition of histamine release by nedocromil is mediated by Anx-A1, it does not require the activation of its receptor FPR2.

To further rule out species-specific variation, similar experiments were repeated in BMDMCs isolated from WT and fpr2/3 null mice. As previously observed, both histamine and PGD_2 release was inhibited by nedocromil in a

concentration-dependent manner in the WT BMDMCs. Whereas, in the fpr2/3 null BMDMCs, nedocromil was unable to inhibit PGD_2 release but was able to decrease the release of histamine across increasing concentrations. These data from both CDMCs and BMDMCs suggest that the inhibition of histamine by nedocromil is dependent on Anx-A1 but is not mediated through FPR2.

A possible speculation for this latter phenomenon could be that Anx-A1 might be interacting with FPR1, which is also another receptor for Anx-A1 (Dufton *et al.*, 2010b). Moreover, nedocromil rapidly causes internalisation of both FPR1 and FPR2 in neutrophils (Yazid *et al.*, 2010b). Another study has shown that the Ac2-26-induced effect on wound closure was blocked by the FPR1 receptor antagonist cyclosporin H (CsH), which also blocked the pro-migratory effects of Ac2-26. However, the peptide WRW4, which is known to antagonize FPR2, did not influence Ac2-26–mediated wound closure. These findings suggest that the pro-migratory effects of Ac2-26 are mediated by FPR1 and not likely mediated by FPR2 (Leoni *et al.*, 2013).

Differential release of pre-formed and *de-novo* synthesised mediators by IgE-dependent and non-immunological stimuli has previously been demonstrated in human mast cells (Benyon *et al.*, 1989; Levi-Schaffer *et al.*, 1989). Another study has shown that inhibition of histamine release is caused by an inactivation of an immediate downstream event following receptor engagement, whereas, key events involved in the inhibitory pathways for PGD_2 release may have higher sensitivity to β-adrenoceptor-derived signals and their inhibitory functions may be longer lasting than those for histamine

release in cultured human mast cells (Tsuji *et al.*, 2004). Such differential signaling events leads to a discrepancy in the mediator release, which could also be explained in this system whereby, FPR2 mediates the release of PGD_2 but not histamine.

Other authors (Church *et al.*, 1987) have noted that some drugs blocks specific release of mast cell mediators and eventually their contribution to the resolution of inflammation. They have shown that sodium cromoglycate inhibits the release of PGD_2 more effectively than that of histamine, therefore reflects a differential inhibition of mediator release.

4.5 Could the MAPK downstream signalling pathway contribute to the 'mast cell stabilising' effects of nedocromil?

The liberation of all mast cell inflammatory mediators is regulated by signaling either after the aggregation of FcεRI by multivalent antigens (Metcalfe *et al.*, 1997; Galli *et al.*, 2005b) or by mast cell secretagogue (Aridor *et al.*, 1990). Although only a small subset of the downstream intracellular signaling pathway of MAPK was tested, the results indicate that Anx-A1 differentially regulates the activation of MAPK in CDMCs pre-treated with nedocromil.

The mammalian MAPKs comprise three major groups, which are the ERKs, the JNKs and p38 that are classified on the basis of their differential activation of agonists, sequence similarity, and substrate specificity (Chang *et al.*, 2001). The activation of ERK1/2 has been linked to cell survival, whereas JNK and p38 are linked to the induction of apoptosis (Xia *et al.*, 1995)

Based on the results, Anx-A1 release induced by nedocromil yielded specific signaling profiles in CDMCs. Compound 48/80 stimulated the expression of both phospho-p38 and phospho-JNK in CDMCs and nedocromil was able to stimulate dephosphorylation of both the MAP kinases. However, in the presence of neutralising anti-Anx-A1 monoclonal antibody, nedocromil was only able to activate p38 but not p-JNK. This suggests that nedocromil suppresses the activation of p38 in an Anx-A1 dependent manner but then this endogenous protein does not play a role in the dephosphorylation of JNK by nedocromil.

Indeed, there is evidence which suggest a differential role for MAPKs in IgE-dependent signaling in human peripheral blood basophils, whereby in contrast to p38 MAPK, JNK is poorly expressed and does not appear to control mediator release (Gibbs *et al.*, 2005). TNF-alpha expression and histamine exocytosis stimulated by exposure of rat peritoneal mast cells to substance P requires the activation of both p38 and JNK MAPKs pathways (Azzolina *et al.*, 2002). Another study has indicated that the release of PGD_2 is mediated through the activation of p38 MAPK pathway in mast cells and macrophages in the skin (Kaur *et al.*, 2013).

Thus far, there has not been any investigation into the ability of nedocromil to influence the MAPKs to exert its inhibitory effects on mast cells. Even though the results presented here are really preliminary, it has certainly opened up possible avenues to study the mechanism of action of nedocromil in regards to the signaling of MAPKs. This could lead to the delineation of a novel-signaling pathway by which Anx-A1 mediates nedocromil to exert its anti-allergic effects on mast cells.

5. CONCLUSIONS

Collectively, the data presented here indicates a novel paradigm for the actions of the anti-allergic drugs involving the anti-inflammatory Anx-A1 protein in mast cells. Initial speculation was that a possible mechanistic relationship existed between anti-allergic drugs and Anx-A1. The anti-allergic drugs that were tested here are from different categories, being either H_1 antagonists, mast cell stabilisers or 'dual-action' drugs. These drugs elicit different pharmacological profiles in respect to the Anx-A1 system, which bears on the question of how they exert their anti-allergic effects.

Indeed, cromones like nedocromil prolongs the ability of GCs to induce Anx-A1 release and thus produce its anti-inflammatory effects. This could be due to the ability of nedocromil to inhibit the phosphatase enzymes, in particular PP2A. It could be speculated that a formulation comprising small doses of GC sufficient to express the Anx-A1 pool within the cell on to the plasma membrane, when combined with a critical dose of nedocromil could lead to superior therapeutic effect with less adverse reactions. Indeed, a new formulation consisting of a combination of azelastine, a 'dual-action' drug and fluticasone propionate, a corticosteroid, has been recently marketed as a nasal spray to treat seasonal allergic rhinitis patients (Kelso, 2008).

The acute mechanism of action of the nedocromil is mediated by Anx-A1. Nedocromil inhibits the release of several mediators from mast cells stimulated with compound 48/80 and does so in an Anx-A1 dependent manner (Figure 5.1). These new findings could potentially greatly enhance

the therapeutic use of cromones, or other compounds with similar mechanism of action, to treat allergic conditions.

The timing of cromone administration relative to the degranulating stimulus is also thought to be crucial (Shichijo *et al.*, 1998) to their effects as these drugs exhibit strong tachyphylaxis (Sung *et al.*, 1977a; Sung *et al.*, 1977b; Church *et al.*, 1987). Indeed, the refractory period that characteristically follows an application of these drugs, led to early speculation that the release of a labile rapidly-depleted anti-inflammatory substance (Thomson *et al.*, 1973) or other transient intracellular phenomenon (Theoharides *et al.*, 1980; Wells *et al.*, 1983) may be involved in their action. The results presented here are consistent with such an interpretation and thus reinforces the concept that the hypothesised 'anti-inflammatory' substance is actually Anx-A1.

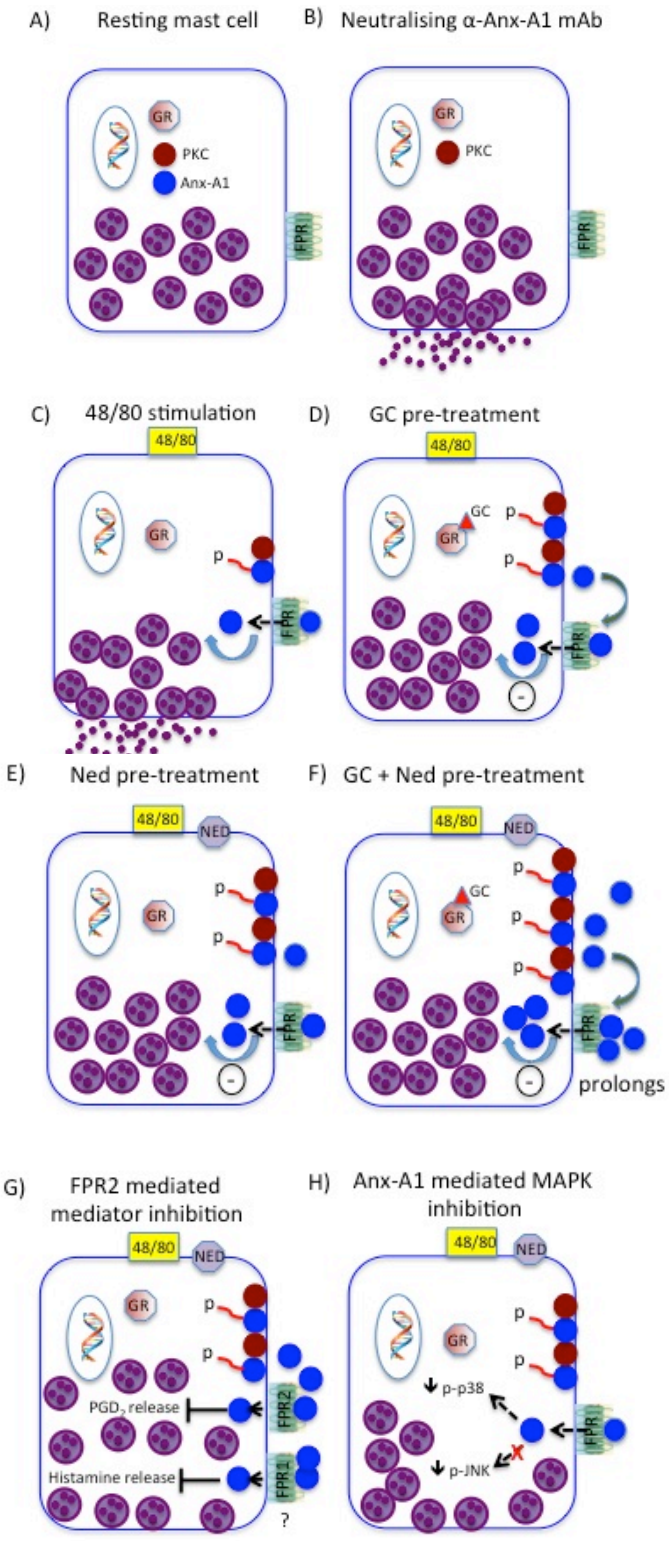


Figure 5.1: Diagrammatic representation of the mechanism of action of drugs treatment in mast cells.

These illustration depicts the pathway by which nedocromil exerts its 'mast cell stabilising' properties in mast cells. A model system in which nedocromil acts through the Anx-A1 protein is proposed. A) In resting mast cells, PKC and Anx-A1 is distributed in the cytoplasm and the granules are intact. B) Upon depletion of Anx-A1, the mast cell undergoes spontaneous degranulation. C) Compound 48/80 stimulation induces the activation of PKC, hence the phosphorylation of Anx-A1, but this action is short-lived and the provoked Anx-A1 exportation is not sufficient enough to inhibit mast cell degranulation. D) Occupancy of GR with GC activates PKC, which in turn enhances the phosphorylation of Anx-A1 and this complex would then be exported out of the cells to act via FPR to inhibit mast cell degranulation. E) Nedocromil potentiates the $PKC_{\alpha/\beta}$ phosphorylation, probably by inhibiting the PP2A enzyme and increases the PKC activation and Anx-A1 phosphorylation and release to exert its 'mast cell stabilising' properties. F) Nedocromil increases the 'dwell' time of PKC activation by dexamethasone, thus increases the $PKC_{\alpha/\beta}$ phosphorylation, which in turn, enhances the Anx-A1 phosphorylation at the Ser^{27} residue. This complex would then be exported out of the cell to act either in an autocrine or paracrine manner via the FPR to exert its anti-inflammatory effects. G) The inhibition of the release of PGD_2 by nedocromil occurs in a FPR2 dependent manner, but the inhibition of histamine release, might be mediated by other FPR members. H) Nedocromil decreases the phosphorylation of p-38 through the Anx-A1 pathway whilst, the dephosphorylation of p-JNK by nedocromil occurs in an Anx-A1 independent manner.

FUTURE PERSPECTIVES

Further extension into this project would provide additional insights into the mechanisms of anti-allergic drugs in regards to the Anx-A1 pathway in mast cells.

Our group has previously collaborated with Dr Andy Vinter of RePharm to determine the molecular docking of nedocromil into the PP2A structure. We hope to pursue further our collaboration to determine the molecular docking of other anti-allergic drugs into the PP2A molecule. This might indicate which anti-allergic drugs interact and thereby prolong PKC activation. The use of PP2A inhibitors would also provide additional data on the mechanistic relationship between these drugs and the phosphatase enzymes.

Since promethazine was observed to have a rather puzzling action apparently being more efficacious without Anx-A1, we would like to explore its potentiating mechanism. One possibility is that in the Anx-A1 null cells the possibility that this drug and Anx-A1 are competing for the same receptor, probably H_4 receptor. This key observation, if confirmed, will further support the possibility that Anx-A1 binds with other GPCRs to exert its anti-inflammatory effects.

Another interesting avenue that could be pursued is the question of co-localisation of S-100 A11 proteins and Anx-A1 in mast cells. It appears that Anx-A1 may reside in mast cells in a complex with S-100 A11 and that the phosphorylation is induced by PKC may cause the dissociation of this

complex, allowing Anx-A1 to localize to the plasma membrane prior to secretion.

In this thesis, some interesting evidence of MAPK signaling pathway elicited by nedocromil in stimulated CDMCs is gathered. We will attempt to further dissect out the signaling profile of other cromones or anti-allergic drugs to understand if indeed Anx-A1 does play a role in this pathway.

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